理學碩士學位論文

히비스커스 메탄올 추출물에 의해 유도되는 casapase independent apoptosis

The caspase independent apoptosis induced by Methanolic extract of *Hibiscus sabdariffa* L.

2014年 8月

서울大學校 大學院

生命科學部

蘇　潭

The caspase independent apoptosis induced by Methanolic extract of *Hibiscus sabdariffa* L.

指導敎授　　鄭 佳 鎭

이 論文을 理學碩士 學位論文으로 提出함

2014年 8月

서울大學校 大學院

生命科學部

蘇　潭

蘇潭의 碩士學位論文을 認准함

委 員 長 _____

副委員長 _____

委　　員 _____

The caspase independent apoptosis induced by Methanolic extract of *Hibiscus sabdariffa* L.

by

Dam Soh

Advisor

Professor Gajin Jeong, Ph. D.

A Thesis for the M. S. Degree in Biological Sciences

August, 2014

Department of Biological Sciences

The Graduate School

Seoul National University

Abstract

The caspase independent apoptosis induced by Methanolic extract of *Hibiscus sabdariffa* L.

Dam Soh
Department of Biological Sciences
The Graduate School
Seoul National University

Hibiscus sabdariffa L. is a polyphenol rich plant that is native to Africa and extract of hibiscus calyces is known for its health beneficial effects in various area inculding antioxidant, antiobesity and anticancer. It is an edible plant that is actually taken as beverages in daily life. Recent novel findings have shown that multiple polyphenols found in extract of hibiscus possess anticancer effect in various cancer cells. It is however still remained unclear how the extract of hibiscus can orchestrate complecate intertwining pathways to induce selective cell death.

In the present study, the anticancer activity of methanolic extract obtained from calyces of *hibiscus sabdariffa* L. (HME) was demonstrated with bio-assays. The apoptotic cell death was selectively induced only for cancer cells but not in normal cells both in vitro and in vivo. Furthermore, HME induced cell death did not exhibit activation of caspase 3, despite the fact that morphological and biochemical characteristics such as DNA fragmentation and mitochondrial dysfunction showed that cells clearly undergo apoptosis.

The mechanisms of which HME mediate apoptosis is through mitochondrial death pathway. Herein, the expression of various pathways were examined in time dependent manner in order to see how HME induced apoptosis is progressed. The activation of both proapoptotic and antiapoptotic proteins in MAP kinases, Akt and Bcl-2 families were evaluated through the course of study. HME induced apoptosis via dual regulation of accumulating proapoptotic proteins such as JNK, p38MAPK, Bim while attenuating antiapoptotic ones. Subsequently, these cascade of signaling activation induced the release of cytochrome c from mitochondria accompanied by mitochondrial dysfunction, which in turn upregulated translocaton of apoptosis inducing factor (AIF) into nucleus. Taken together, these findings indicated that signaling cascades induced from MAPK and Akt pathways altered mitochondrial membrane potential via mediating Bcl-2 family protein activation and releasing cytochrome c. As a consequence, the translocation of AIF played crucial role in HME-induced caspase independent apoptosis in human leukemic U937 cell.

Keywords : Anticancer, Lukemia, Hibiscus sabdariffa L., Caspase independent apoptosis.

Student Number : 2012-20313

CONTENTS

List of Figures

List of Abbreviations

AIF	Apoptosis inducing factor
ELISA	Enzyme Linked ImmunoSorbent Assay
ERK	Extracellular signal-regulated kinase
FADD	Fas Associated Death Domain
HBSS	Hank's Balanced Salt Solution
HME	Hibiscus Methanolic Extract
JNK	c-Jun N-terminal kinases
MAPK	Mitogen-activated protein kinases
PBS	Phosphate Buffered Saline

Chapter Ⅰ. Introduction

1. Natural polyphenols

Polyphenols are the largest group of phytochemicals that are characterized by the presence of multiple phenol ring structures. Dietary phenolics are highly diverse and the chemical and physical properties of polyphenols are largely decided by their aromatic structures. Four major subgroups of polyphenols are phenolic acids, flavonoids, polyphenolic amide and other polyphenols (Tsao, 2010).

Natural polyphenols are widely found in various plants and there are growing interests in these compound due to their various health benefits such as antioxidant, antimicrobial and anticarcinoma effects (Engelhardt *et al.,* 2000). In addition, recent study suggest that taking polyphenol-rich diet would help to prevent number of cardiovascular diseases, such as high blood pressure, obesity and diabetes (Lecour *et al.*, 2011). There are many novel findings about molecular mechanisms of polyphenols and it has been proposed that the ring structures of polyphenol compounds possess high binding affinity for proteins, so that they can form either soluble or insoluble protein-polyphenol complex (Papadopoulou *et al.,* 2004). Previous studies demonstrated that some flavonoids can act as signaling molecules that displays high degree of specificity to number of kinases (Gibbins *et al.*, 2013). These polyphenol compounds are synthesized in almost every natural plants and their composition and amount varies greatly.

2. Hibiscus sabdariffa

Hibiscus sabdariffa L. (Malvaceae), common name roselle, is characterized by its red colored calyces and ampule amounts of polyphenol contents. It is a polyphenol-rich plant that is widely consumed by many people around the world in many different ways, such as herbal tea, food dye, and folk medicine. There are also growing businesses of hibiscus farming industry due to its demands. Therapeutic potentials of hibiscus calyces include antimicrobial, antitumor, cardioprotective, chemoprevention, antihypertensive, antioxidant, and hypocholesterlemic activities. Despite the fact that hibiscus contains plentiful natural polyphenol compounds that are known as anticancer agent, there are not much ongoing studies about anticancer effect of hibiscus extract. The major pholyphenol compound found in hibiscus calyces are protocatechuic acid, catechin, epigallocatechin(EGC), epigallocatechin gallate(EGCG), and caffeic acid (Wang *et al.,* 2005). In addition, there are several flavonoids such as gossypetin, hibiscetin, hibicitrin, quercetin and other anthocyanins (Tseng *et al.,* 2000). Many of these compounds are known to have either antioxidative or anticancer effect and some of these molecules have showed synergetic effects as well. However, it is interesting that current researches about these pharmacological effects of hibiscus are more focused on the effect of each polyphenol compound rather than extract of whole calyces even though that is what used as dietary source.

The anticancer ability of hibiscus extract was shown in many different cancer cell types through various pathways (Wang *et al.,* 2005). Another data indicate that the extract of hibiscus calyces induce apoptosis through both intrinsic and extrinsic pathway in

prostate cancer cell (Chen *et al.,* 2012). Similarly, the methanol extract of hibiscus calysis could induce apoptosis in human promyelocytic leukemia cells through activation of JNK/p38 MAP kinases and it was also excuted through both mitochondria and receptor death pathway (Wang *et al.,* 2007). The mechanism by which hibiscus polyphenols can induce an anticancer effect is by regulating various signaling pathways that involve in cell survival. Several data indicate that hibiscus polyphenols can mediate apoptosis through regulating Bcl 2 family proteins. It is said that, the abnormal upregulation of Bcl−2 protein can cause impaired apoptosis in leukemia, thus, knowing that hibiscus extract has ability to control Bcl−2 family proteins would be worth noting (Xu *et al.,* 2013). According to previous studies, it seems clear that mitocondrial pathway and Bcl−2 family proteins play major role in cell apoptosis induced by hibiscus extract and as previously mentioned, MAP kinases also plays a key role to control it, and yet, how these two pathways interact in apoptosis induced by the extract of hibiscus remained unclear.

3. Leukemia

Leukemia is a type of cancer that occurs when immature white blood cells are in state of uncontrolled cell growth and multiplication. The term, leukemia, derives from the Greek words leukos "white" and haima "blood". The blood cells are mainly composed of three types cells: red blood cells(erythrocytes), white blood cells(leukocytes) and platelets. Abnormal growth of immature white blood cells that are incapable of normal function, interfere with normal white blood cells by out competing them in numbers. In addition, accumulation of leukemia cells eventually disrupt normal blood production and result in anemic state.

3. 1. Acute Leukemia

Cases of leukemia can be first classified into two large categories, acute or chronics. Acute leukemia is characterized by chaotic multiplication of blood cells before immature stage. Notable cellular features comprise a rapid decrease of mature granulocytes, erythrocytes and platelets. Acute leukemia can be further divided into lymphocytic leukemia and myelogenous leukemia. This division depends on which cell type of blood become transformed. When acute leukemic transformation occurs to the type of cells that are precursor to lymphocytes, it is called acute lymphoblastic leukemia (ALL), which is the most common type of leukemia among young patients. While when transformation takes place on other types of marrow cells such as erythrocyte, promyelocytes, monocytes and platelets, it is called acute myelogenous leukemia (AML).

3. 2. Chronic Leukemia

Progression of chronic leukemia is rather slower, thus allows cells to more mature but still abnormal. Although accumulation of abnormal blood cells are slower than that of acute leukemia, the rate of production of leukemic cells are faster that normal cell production and eventually they outnumber the normal cells. This is the type of leukemia that is found more often in elder patients. As mentioned earlier, the chronic leukemia can be further divided into lyphocytic and myelogenous as well.

3. 3. Treatment

The treatment of leukemia is limited with certain chemotherapy and radiations. However the chance of relapse is quite high and once relapsed, the tumor can become resistant to the previous medications. In fact, during the clinical research, about 70% of chronic lymphocytic leukemia(CCL) patients showed relapse and 70% of them became resistant to previous existing therapy (Russo *et al.,* 2011). The most common characteristic of human leukemia is known to be the overexpression of antiapoptotic Bcl-2 family protein especially Mcl-1. Recent studies showed that increase in antiapoptotic gene *mcl-1* or post-translational stablization of Mcl-1 protein is essential for the development and sustained growth in leukemia (Glaser *et al.,* 2012). Thus, there are many ongoing researches on treating leukemia that are now targeting Mcl-1 downregulation.

4. Cell Death

The morphological and biochemical classification of cell death can be divided into three large categories: apoptosis (Type I), autophagic cell death (Type II), and necrosis (Type III). Dying cells are engaged in several processes until they reach "point of no return" stage, where processes can not be reversible (Kroemer *et al.,* 2009). Unlike necrosis, which shows distinctive morphological characteristic, it is not clear to fully distinguish autophagic cell death from apoptosis since their intrinsic mechanisms share many same pathways (Lockshin *et al.,* 2004).

4.1 Apoptosis, a programmed cell death

Apoptosis is a programmed cell death critical for the cell maintenance and normal development of multicellular organisms (Wyllie *et al.,* 1980). In course of apoptosis, extracellular stress stimuli causes activation of series of pathway cascades that induce self destruction. The term "apoptosis" is first coined in 1972 by Kerr *et al.* Common morphological characteristics of apoptosis are cell shrinkage, blebbing, rounding and blebbing of nuclei with condensation of chromatin and apoptotic body formation (Lockshin *et al.,* 2004). Formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances that form on the cell surface (Kerr *et al.,* 1972). Biochemical feature of apoptosis includes DNA fragmentation that causes to create oligonucleosomal "ladder", mitochondrial membrane potential distruption, and proteolytic cleavage of intracellular substrates. As opposite to necrosis, which causes distruption of cell membranes and organelles and result in

massive cellular inflammatory response, cells undergo apoptosis break into several apoptotic bodies and gradually degrade, thus prevent an inflammatory response. Causes of cell death by apoptosis can be various, including starvation, cellular DNA damages by oxidants, irradiation or glucocorticoids (Cohen and Duke, 1992). It is interesting that apoptosis can also occur independent from cellular stress. Most obvious case is the digit formation during embryonic development of mouse and apoptosis during the metamorphosis of a tadpole. The regulation of cell death is critical within the immune system in order to prevent autoimmune disease. Self-reactive lymphocytes need to eliminated from immune repertoire so immature lymphocytes are susceptible to apoptosis (Boise *et al.,* 1995). Taken all together, apoptosis is crucial in maintaining cellular homeostasis.

4. 2. Apoptosis and Cancer

Defective apoptosis result in uncontrolled growth of abnormal cells, is the major causative factor in the development of cancer. There are multiple mechanisms that allow tumor cells to avoid entering normal apoptotic processes. Common mechanisms include overexpression of antiapoptotic proteins, attenuating the apoptosis regulatory molecules and altering surveillance proteins of normal cell cycle.

4. 3. Mechanisms of Apoptosis

The apoptotic process is triggered by two major pathways: receptor initiated extrinsic pathway and mitochondria mediated intrinsic pathway. The extrinsic apoptotic pathway is induced by members of the death receptor superfamily such as Fas(CD95).

Upon death signal, Fas ligand(FasL) binds to receptors, which then leads to the formation of a death-inducing complex by recruiting the adaptor molecule Fas-associated death domain (FADD) and pro-caspase-8 (Wang *et al.*, 2004). While extrinsic pathway initiated by exterior stimuli, intrinsic pathway is controled by pro- and anti-apoptotic members of Bcl 2 superfamily. After signal is sent from either pathway, the death inducing signals from activated death receptors and induce activation of initiator caspases, then caspase 3 cleavage follows to fully induce apoptosis to dying cell (Jung *et al.*, 2009).

4. 4. Caspase independent apoptosis

Classical concept of apoptosis is a caspase-dependent form of cell death. It has been known that activation of a family of cyteine-dependent aspartate-directed proteases, caspase, is critical in apoptosis process (Chinnaiyan and Dixit, 1996). However, recent novel findings have shown that apoptosis can be induced in absence of caspase activation. The central player of caspase independent apoptosis is known as apoptosis-inducing factor (AIF). In nonapoptotic cell, AIF is localized in mitochondria but when apoptotic signal causes the mitochondrial dysfunction, it is released from mitochondria and translocate into nucleus and starts to trigger apoptosis.

5. Cell signaling pathways involved in cell apoptosis

5.1 MAPK

In general, the role of MAP kinases is the transmission of extra-cellular stimuli from the cell membrane to nucleus through cascade activity. Cells are continuously required to respond to external stress signals and decide whether 5to continue to go on or suicide. Mitogen activated protein kinases (MAPKs) are serine/threonine/tyrosine-specific protein kinases that are crucial for regulating the fate decision of cell. In response to stimuli, MAP kinases are involved in various cellular responses including inflamation, cell survival, differentiation and apoptosis. Activation of MAP kinases start from phosphorylation of serine/threonine residues on MAPK kinase kinase (MAPKKK). Once MAPKKK is activated, it phosphorylates threonine/tyrosine residue of MAPK kinase (MAPKK), which in turn induces the activation of MAPK (Ge *et al.,* 2003).

5.1.1 ERK/MAPK

There are three large subfamilies of MAPKs: extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38-MAPK. ERKs are composed of its own family members, ERK1 and ERK2, and they are well known for involving in cell proliferation and survival response to growth stimuli. ERKs are activated when a threonin and tyrosine residues are phosphorylated by MEK1 and MEK2. Once ERKs are activated, they translocated to

the nucleus and regulate transcriptions by phosphorylating several transcriptional factors and substrates. In most of times, ERKs mediate prosurvival regulation by mediating upregulation of other anti-apoptotic proteins.

5.1.2 JNK/SAPK

JNKs are stress-activated protein kinases that belong to superfamily of MAP kinases. The role of JNKs can be varied from regulation of cell proliferation, differentiation and apoptosis. Initially JNKs are identified as UV-responsive protein kinases but several studies showed that JNKs can respond to various stimuli and mediate number of different cellular responses. Yet, it is clear that the most obvious role of JNKs are mediating apoptosis under cellular stress. Activation of JNKs are induced by two of MAPK kinases, MKK4 and MKK7, via phosphorylation of threonine and tyrosine dual residues. Once activated, JNKs can induce apoptosis through upregulation of pro-apoptotic genes or by modification of mitochondrial proteins.

5.1.3 p38 MAPK

The family of p38 mitogen-activated protein kinases are the last subfamily of MAPK superfamily. Like the other members of MAPK, they involve in regulation of various cellular function under different stimuli. Especially, p38 MAPKs are activated by stress stimuli and they play central role in controlling cell death (Porras *et al.*, 2004).

There are four proteins that make up p38 MAPK: p38α, p38β, p38γ and p38δ. Among these four members, p38α is the most abundant and well known (Mittelstadt *et al.*, 2005). p38 MAPKs are mainly phosphorylated and activated by the upstream MKK3 and MKK6 kinases, although MKK4 can sometimes activate p38α (Cuenda *et al.,* 2007). Since MKK4 also activates JNK pathways, p38 MAPKs and JNKs are often stimultaneously activated (Cuevas *et al.*, 2007). p38 signaling pathways are involved in apoptosis in the way similar to that of JNKs. The crosstalk between p38 and JNK pathways during apoptosis induction has been frequently reported. The expression of JNKs regulate the activation of p38 indirectly through downregulation of Jun (Stepniak *et al.*, 2006). In addition, p38 MAPKs can negatively regulate JNK activity at the level of downstream (Perdiguero *et al.*, 2007). This coordinated activation of two pathways can regulate cell function effectively.

The mechanisms of which p38 MAPKs can induce apoptosis through mediating different signaling pathways, include the regulation of expression and activity of Bcl−2 family.

5.2 Bcl-2 family pathway

Bcl-2 family proteins are apoptosis regulators that govern mitochondrial outer membrane permeabilization (MOMP) and mediate mitochondrial intrinsic apoptosis pathway. In mitochondrial death pathway, the role of Bcl-2 family proteins play central role as regulators of cell survival and their activation is tightly mediated by MAPK proteins. Two of major Bcl-2 family proteins that are known to mediate intrinsic apoptosis are BAX and Bim. BAX, Bcl-2-associated X protein, is an apoptotic regulator that promotes apoptosis by blocking antiapoptotic proteins, Mcl-1 and Bcl-2 (Marzo *et. al.,* 2013). BAX is regulated by p38 through complex mechanisms, so that the level of BAX can be upregulated by p38 (Porras *et. al.,* 2004). Upon various stimuli, p38 can mediate translocation of BAX to mitochondria. Another Bcl-2 family protein, Bim, is also regulated by p38 (Cai and Xia, 2008). In addition, accumulation of Bim can mediates translocation of Bax, since Bim and Bax complexes are formed in cytosol and translocate to mitochondria during apoptosis (Marzo *et. al.,* 2013).

5.3 PI3K/Akt pathway

PI3K/Akt pathway is an intracellular pathway that involves most notably in cell proliferation. Akt is serine/threonine kinase that is tightly regulated by PI3K. Although Akt is involved in various cell functions such as cell growth, proliferation, survival and even apoptosis, activation of Akt generally mediates cell survival via downregulation of proapoptotic molecules such as Bim. There are evidences of synergistic effect of Akt and ERK can intervene the apoptosis process in human leukemic cells (Dong *et al.*, 2007). This attenuation of Bim then leads to overexpression of antiapoptotic Bcl-2 family proteins such as Bcl-2 and Mcl-1.

6. Purpose of study

The purpose of this study is to find out the mechanisms of which hibiscus extract induce the cell death in human leukemia cells. Since it seems that extract of hibiscus involves in various pathways that mediates cell death, it is important to find out the clear molecular pathway and how it orchestrate this process. Also unlike with classical findings, this study showed that methanolic extract of hibiscus was able to regulate cell apoptosis in caspase independent way, probably because of its polyphenol composition. Furthermore, hibiscus plants are cultivated in many different regions and so it is possible that they might have different chemical compositions and these difference could even create new synergetic effects.

In addition, *Hibiscus sabdariffa* is commercial plant that its demand is growing in worldwide because it is relatively easy to grow and can be stored for long time as dried form and still its nutritional value remains. The calyces of hibiscus flower can be used in various ways in our life since its bright red color can be used food coloring or natural synthetic dye and not to mention that it can always be taken as beverage as well. Until now, even though it is already used as folk medicines in some local areas, the therapeutic ability of hibiscus is still not well known. However, once it is proven to have a pharmacological possibility, its value would expand further.

Chapter Ⅱ. Materials and Methods

1. Maitenance of cell lines and culture media

1.1. Cell line

Cell lines used in the present study are murine macrophage RAW 264.7, murine colon carcinoma CT 26, murine fibroblast 3T3, human T lymphocyte Jurkat, human acute monocytic leukemia THP-1 and human luekemia U937 cells were obtained from Amerian Type Culture Collection (ATCC, Manassas, VA, USA). The culture medium used for U937 cells throughout this study was RPMI-1640 (Gibco-Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS (Hyclone, Logan, UT) and 20 μg/ml of gentamycin (Sigma Chemical Co., St. Louis, MO, USA). Cells were maintained between $1x10^5$-$1x10^6$ cell/ml in sterile plates and cultured at 37°C in a humidified 5% CO_2 incubator. Cells were collected and washed with HBSS for 2 times and resuspended in complete medium

1.2. Isolation of monocytes from human peripheral blood

Fresh peripheral blood was obtained and placed with heparin for the brief moment while assay was prepared. Then using a Ficoll-Hypaque density gradient method, the mononuclear cells were removed. Mononuclear cells were cultured by standard methods and monocyte-derived macrophages were obtained by adherence.

1.2.1. Hank's Balanced Salt Solution (HBSS, pH 7.2)

CaCl2	0.14 g
KCL	0.4 g
KH2PO4	0.06 g
MgCl2	0.1 g
MgSO4·7H2O	0.1 g
NaCl	8 g
NaHCO3	0.35 g
Na2PO4	0.09 g
Dextrose	1 g
Phenol Red	0.01 g
T.D.W.	1 L

2. Preparation of Hibiscus extract.

Hibiscus methanolic extracts (HME) were prepared from the freeze-dried flower of *H. sabdariffa* L. Each 5g of hibiscus powder was extracted in 500ml of different solvents for 24 hours at 4°C: distilled water, 30% ethanol, ethanol, and methanol containing 1% HCl. The extract was then filtered and concentrated at 60°C bunder vacuum using rotary evaporator (Roche, USA). The precipitate was then dissolved in PBS and stored at -20°C before use.

Phosphate Buffered Saline (PBS, pH 7.2)

NaCl	8g
KCl	0.2g
KH2PO4	0.2g
Na2HPO4*H2O	2.89g
D.W	1L

3. Sample analysis of HME.

3.1 Total phenol content assay

Total phenol content (TPC) was measured with folin ciocalteu method. Gallic acid was used as standard. 0.5mg/mL stock standard solution of gallic acid was prepared by dissolving 500mg of dry gallic acid in 10mL of extracting solvent and then diluted 100 times with distilled water. Working standards of between 0.01 and 0.05 mg/mL were prepared by diluting the stock solution with distilled water. The extract was prepared at concentration of 0.1mg/mL to 2mg/ml. 100μL of extract was transferred into a test tube and mixed with 0.75mL of 10-fold diluted Folin-Ciocalteu reagent. Then, 0.75mL of 6% (w/v) sodium carbonate was added to the tube. The mixture was then incubated at room temperature for 90 minutes and absorbance was read at 725nm.

3.2 Quantitative analysis of H. sabdariffa by HPLC

The quantitative and qualitative analyse of metanolic extract of HME was performed with High Performance Liquid Chromatography (HPLC). Polyphenol standards, quercetin, galic acid, caffeic acid, epigallocatechin gallate(EGCG), epigallocatechin, catechin and protocatechuic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA), To prepare a standard marker substance solution, each standard polyphenols were dissolved in pure ethanol to give various concentrations within the range of 100-1000 ppm. Then the freeze dried powder of HME sample was dissolved in 1% HCl-methanol and filtered though 0.22μm nitrocellulous membrane filter and aliquots of 10μl were injected for analysis. The HPLC

system used for analysis was Ultimate 300 (Dionex, USA) with an isocratic analytical pump (Dionex LPG-3400SD, USA), a UV/Vis Diode Array Detector (Dionex VWD-3100, USA), and system controller. Flow rate was set to 1ml/min and run time was 40 minutes.

4. Cell viability assay

Both U937 cells and normal human lyphocytes were seeded at a density of $1x10^5$ cells/well and cultured with various concentrations (0-4mg/ml) of HME and incubated for 24hours. In the case where inhibitor was treated, cells were incubated with caspase inhibitor for 2hours and HME was treated for appropriate hours. Then 10%(v/v) of 3-(4,5-dimethylthiazol-zyl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated another 4 hours. After incubation, media was removed and the pellets were dissolved in isopropanol and measured absorbance at 563nm. The percentage of viable cells estimated by comparison with untreated control cells.

Treatment of individual polyphenols were followed by calculating amount of each polyphenols in HME. Since the quantitative analysis showed that there are 40.36% gallic acid, 4.4% caffeic acid and 40.52% Quercetin. Therefore the amounts of each polyphenols was calculated by each percentage of various concentration of HME.

5. Colorimetric TUNEL assay

The detection of DNA fragmentation of cells treated with 2mg/ml of HME for 24 hours was observed by DeadEnd colorimetric TUNEL assay (Promega, USA) Evaluating the apoptosis via end-labeling of fragmented DNA had been used as simple and effective method. The cells were prepared with a density of $1x10^5$ cells/ml and incubated with 2mg/ml of HME and allowed to grow for indicated time periods. After incubation, cells were collected washed by centrifugation once and resuspended in fresh PBS. Cells were then placed on poly-L-Lysine-coated slide and fixed by immersing in 4% paraformaldehye solution in a coplin jar for 25 minutes at room temperature. The slide was washed by immersing in fresh PBS for 5 minutes twice and cells were permeabilized by immersing the slide in 0.2% Triton X-100 solution for 5 minutes at room temperature. The slide was rinsed with PBS again and the excess liquid was removed. After, cells were equilibrated with equilibration buffer for 10 minutes and rTdT mix that include Biotinylated nucleotide and rTdT enzyme was treated and the slide was incubated at 37°C for 60 minutes inside a humidified 5% CO_2 incubator. The reaction was terminated by immersing slides in SSC (0.15M NaCl and 0.02M sodium citrate) for 15 minutes at room temperature and endogenous peroxidases were blocked with 0.3% hydrogen peroxide for 5 minutes. Next, the slide was washed with PBS again and streptavidin HRP solution was added to the slide and let 30 minutes in room temperature. Lastly, the DAB solution was added to the slide and stained slide was observed using a light microscope.

6. Mitochondrial membrane potential assay

The mitochondrial membrane potential was measured with tetraethylbenzimidazolylcyanin iodide (JC-1). JC-1 is a cationic dye that accumulates in energized mitochondria to measure the mitochondrial membrane potential. U937 cells were grown in RPMI with the density of approximately $1x10^6$ cells per plate. In order to test toxicity, 2mg/ml of hibiscus sample was treated to cells and incubated for 0-24 hours. For positive control, cells were treated for 4 hours with 100μM of FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone). After incubation, cells were collected and washed by centrifugation twice in PBS. Cells were then resuspended in 500μl of the JC-1 solution and incubated at 37°C for 30 minutes in a humidified 5% CO_2 incubator in the dark. Cells were again washed by centrifugation with 1ml of dilution buffer and resuspended in fresh dilution buffer with density of $1x10^6$ cells/ml. Then 50μl of suspended cells were seeded on 96-well dark sided plate for reading. Depolarization of mitochondria membrane was measured by reading end point in the presence of buffer on a fluorescent plate reader (FlexStation, USA) The excitation wavelength was set at 535nm and 475nm and emission wavelength was set at 530nm. At low mitochondrial membrane potential, JC-1 exist as monomers that yields green fluorescence with emission of 530nm and at high membrane potential, the dye aggregates yielding red emission. The decrease in mitochondrial membrane potential was measured by ratio change in red fluorescence over green.

7. Preparation of cell lysates and western blot analysis.

U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HE for various time periods (0-24hrs). Cells were then washed with ice cold PBS for two times and incubated with cold lysis buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl, pH 7.5) for 15 mins, followed by centrifugation at 10,000g for 10min at 4°C. Then the supernatant was collected and stored at -80°C before use.

Protein concentration was measured with BCA assay and 20μg of protein was mixed with sample buffer (0.4M Tris-HCl, pH 6.8, 0.5M dithiothreitol, 10% SDS, 50% glycerol and 0.005% bromophenol blue) and boiled for 5 mins. Equal amounts of lysate protein were run on 10-20% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Invitrogen, USA). After transfer, the membrane was blocked with appropriate blocking buffers and incubated on the shaker for 2hours at room temperation. Then the membrane was washed three times with TBST (500mM NaCl, 20mM Tris-HCl (pH 7.4), and 0.1% Tween 20) and incubated with specific primary antibody for overnight at 4°C. Finally, the blot was incubated with HRP-conjugated secondary antibody for 2 hours at room temperation and membrane was developed with ECL solution (Promega, USA) for image analysis with Chem-doc (Fusion-SL Chemiluminescence system, Fisher Biotec. AU).

Chapter Ⅲ. Results

1. Analysis of *Hibiscus sabdariffa* L.

1.1. *H. sabdariffa* L. extraction and total phenol content

Freeze dried powder of *H. sabdariffa* L. (5g) was extracted with 500ml of each distilled water, 30% ethanol, ethanol and 1% HCl containing methanol for 24 hours at 4°C. The extract was then filtered and concentrated with rotary evaporator. The precipitation was collected and dissolved in PBS and stored −20°C before use.

Total phenol content (TPC) was measured with folin ciocalteu method, which is briefly that polyphenols in plant extracts react with Folin−Ciocalteu reagent, a redox reagent, to form a blue complex. TPC of each extract was 2675mg/ml, 3043mg/ml, 3587mg/ml, and 5645mg/ml (Figure 1). Since the TPC of methanol extract was highest, the 1% HCl containing methanol extract was used in the rest of experiments.

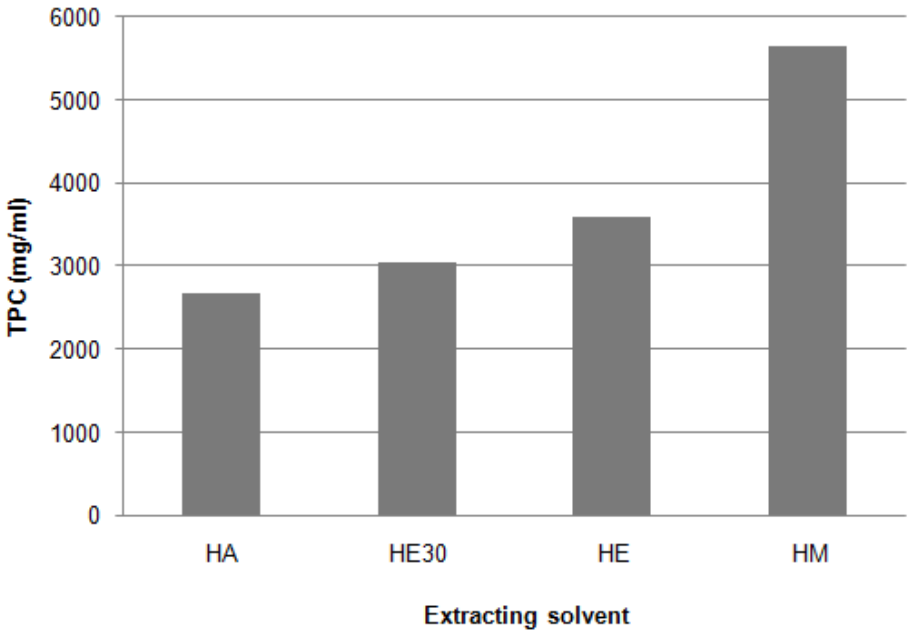


Figure 1. Total phenol contents. The total phenol contents of different hibiscus extract samples were measured using gallic acid as standard (mg/L). (HA; Aqueous extract, HE30; 30% ethanol extract, HE; pure ethanol extract, HM; 1%HCl-methanolic extract). Values are mean ± S.E.M

1.2. Quantitative analysis of polypenol compounds in HME

The polyphenol contents of hibiscus methanolic extract (HME) was analyzed by HPLC system. Representative chromatogram (280nm) and UV spectrum of quercetin, caffeic acid, galic acid and other polyphenols were acquired using HPLC. According to known spectra from existing standard and retention time. Four major polyphenols that had been mentioned to be found in hibiscus extract, protocatechuic acid, catechin, epigallocatechin (EGC), epigallocatechin gallate (EGCG), were not found in HME. Instead the major polyphenol components found in HME was identified as galic acid, caffeic acid and quercetin and the amount of each polyphenols was calculated as 40.36%, 4.4%, and 40.52% (Figure 2 and 3).

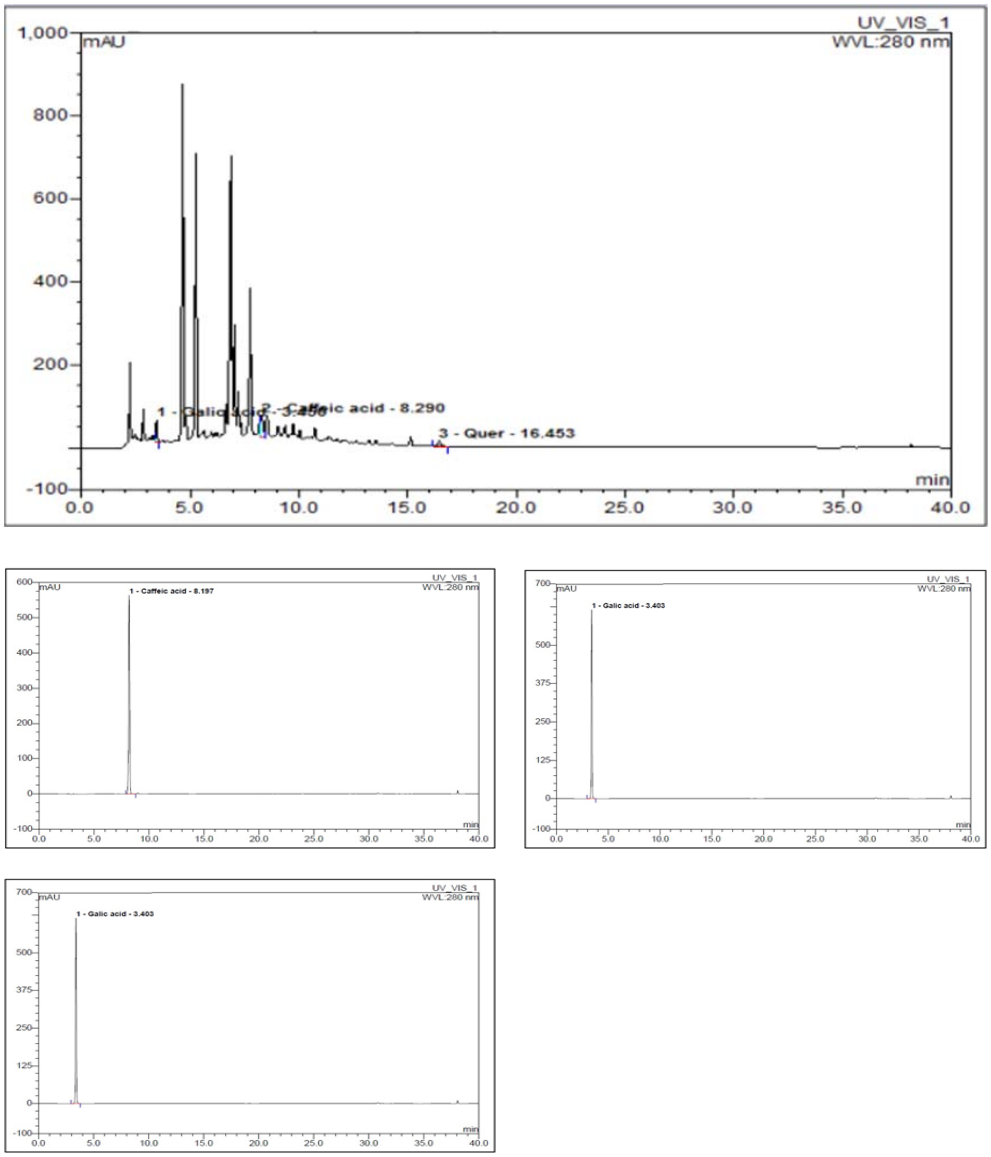


Figure 2. HPLC chromatogram of hibiscus methanol extract. (A) HPLC chromatogram of hibiscus extract in 1% HCl methanol(HME). The contents found was gallic acid, caffeic acid, and quercetin. (B) Caffeic acid, gallic acid, and quercetin standards. (UV_VIS_1, wavelength 280)

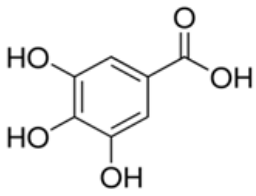
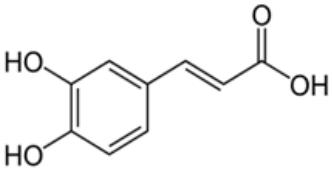
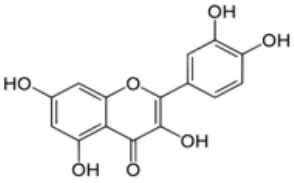
Name	Chemical Structure	M.W. (g)	Ratio in raw material
Gallic acid 3,4,5-trihydroxybenzoic acid		170.12	40.36%
Caffeic acid 3-(3,4-Dihydroxyphenyl)-2-propenoic acid		180.16	4.4%
Quercetin 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one		302.236	40.52%

Figure 3. Structures of gallic acid (GA), caffeic acid (CA) and quercetin (Q) derived from *Hibiscus sabdariffa* extract. The contents of each compounds in HE were 40.36%, 4.4%, and 40.52% respectively.

2. Selective cytotoxicity of HME on cancer cells

2.1 HME selectively induces cell death on various cancer cell lines.

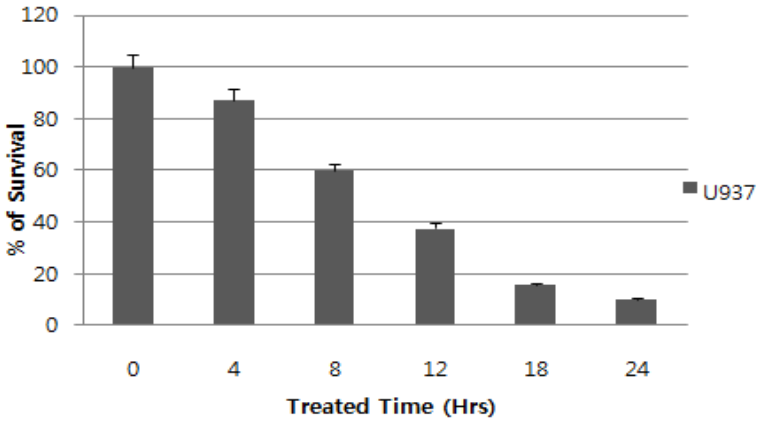
To test hibiscus extract have cytotoxic effect againt cancer cells but not in normal cells, hibiscus extracts were treated to both human leukemic U937 cells and normal human lymphocytes. The dose dependent effect of hibiscus extract on U937 cells evaluated at 0−4mg/ml. While time dependent effect was measured for 0−24 hours with concentration of 2mg/ml. The cell viability was measured with the optical density of formazan formed in cells and calculated as follows:

$$\text{Cell viability (\%)} = \frac{O.D.treatment}{O.D.control} \times 100$$

As a result, hibiscus extract showed cytotoxicity against U937 cells in both time and concentration dependent manners (Figure 4A and B) while it didn't affect the viability of normal cell (Figure 5). Also HME showed about 50% cytotoxicity on U937 cells at the concentration of 2mg/ml.

To assess the effect of hibiscus extract on inhibition of various cancer cell lines including THP−1, CT−26, Raw 264.7, 3T3 fibroblast and Jurkat cells, each cell lines were treated with hibiscus extract for 24 hours and viabilities were measured by MTT assay (Figure 6). The result indicates that hibiscus extract possess cytotoxic effect on all of tested cell lines suggest that hibiscus extract is associated in general apoptotic pathway of cancer cells.

(A)



(B)

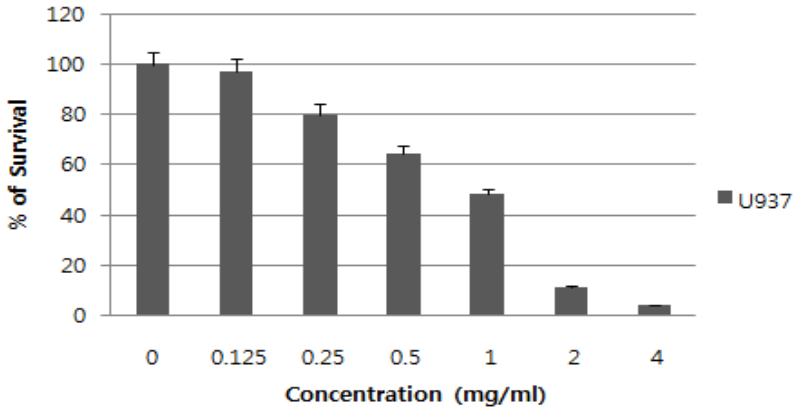


Figure 4. Cell cytotoxic effect of HME on U937 cells. (A) U937 cells ($1x10^5$ cells/well) were treated with HME for 24 hours with various concentrations. (B) U937 cells ($1x10^5$ cells/well) were treated with 2mg/ml of HME for indicated time periods. Cell viability was then meausured by MTT assay.
Cell Viability (%) = (O.D. Treatment / O.D Control) x 100.
Values are mean ± S.E.M

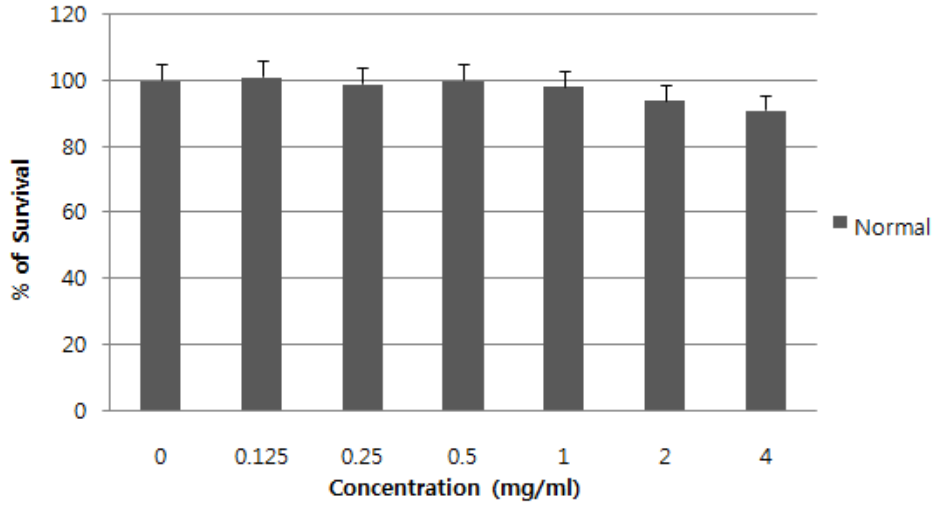


Figure 5. Cell cytotoxic effect of HME on normal human lymphocytes. Human lymphocytes ($1x10^5$ cells/well) were treated with hibiscus extracts for 24 hours with various concentration, respectively, and cell viablity was measure by MTT assay.
Cell Viability (%) = (O.D. Treatment / O.D Control) x 100.
Values are mean ± S.E.M

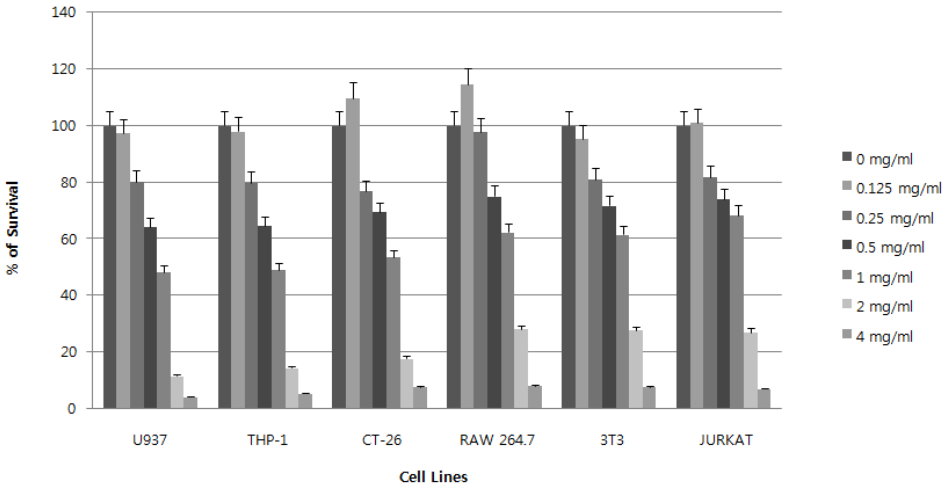


Figure 6. Effect of HME on survival of various cell lines. Each cell lines ($1x10^5$ cells/well) were treated with various concentrations of hibiscus extracts for 24 hours. Cell viability was then measured by MTT assay. Values are mean ± S.E.M.

2.2. Effect of individual polyphenol compounds on U937 Cell

To test whether the cytotoxicity of HME was induced by specific polyphenol compound, each polyphenol was treated to U937 cell with various concentrations for 24 hours. The result showed that individual polyphenol was able to induce cell death only in concentrations over 10μM (Figure 7A), while the amounts of these polyphenols in 2mg/ml of hibiscus extract were less than 1μM.

The synergistic effect of polyphenol compounds were also tested. First, two of each polyphenol with equal amounts were treated to cells for 24 hours then all three polyphenols were treated to cells and also incubated for 24 hours. The result indicated that synergetic effect of each polyphenol was strongest in gallic acid and quercetin mix. Interesting observation was that the cytotoxic effect of all three polyphenol cocktail was less powerful then that of gallic acid and quercetin mix (Figure 7B).

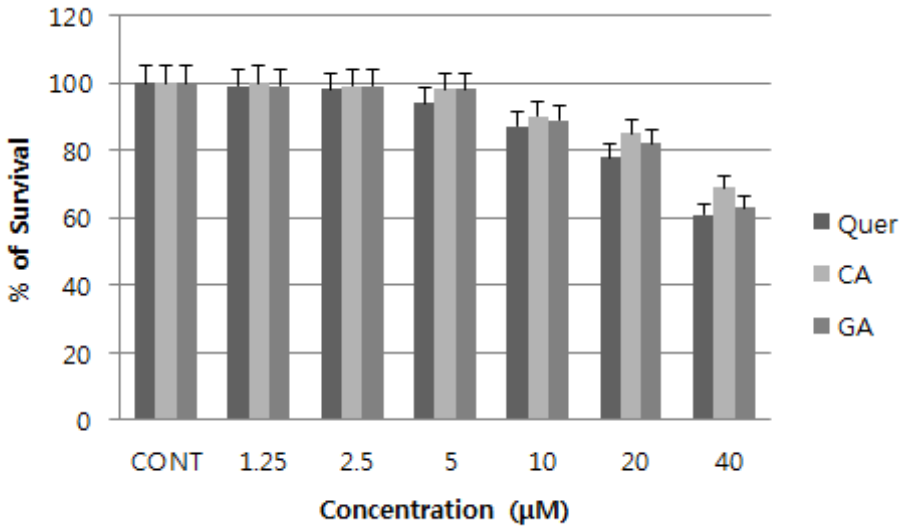


Figure 7. Cytotoxicity of individuals polyphenol against U937 measured by MTT assay. (A) U937 cells($1x10^5$ cells/well) were treated for 24hrs with various concentrations of each polyphenols. (GA: Gallic acid, CA: Caffeic acid and Quer: Quercetin). Values are mean ± S.E.M

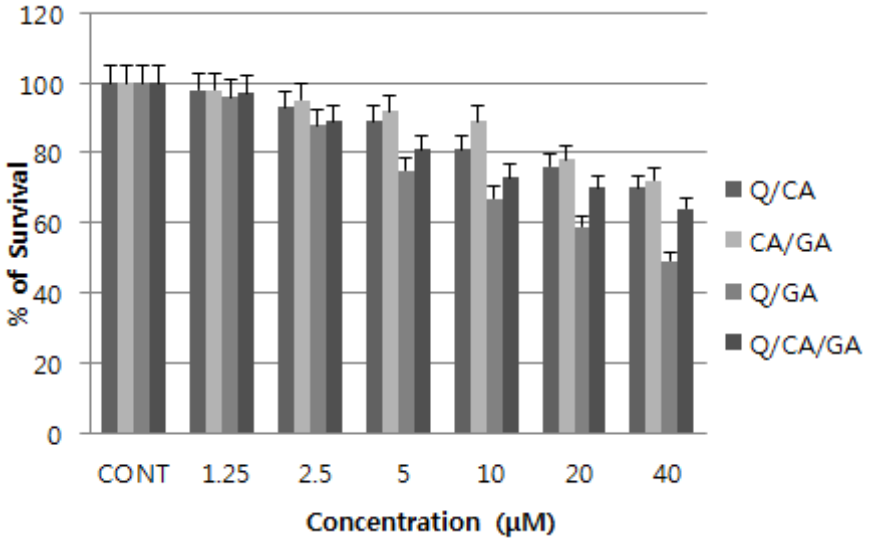


Figure 7. Cytotoxicity of individual polyphenols against U937 was measured by MTT assay. (B) U937 cells($1x10^5$ cells/well) were treated for 24hrs with various concentrations of two or three polyphenols together. (GA: Gallic acid, CA: Caffeic acid and Q: Quercetin). Values are mean ± S.E.M

2.3. Cytotoxic effect of HME in vivo

Cytotoxic effect of hibiscus extract was also tested in animal model. In order to test cytotoxic effect of hibiscus extract, total of twenty female mice were divided into four groups of five. Each group was fed with unlimited amount of solid forage and water.

During the experiment, cytotoxicity of hibiscus extract was tested by peritoneal injection every other day. Control group (group 1) was injected with sterilized PBS and group 2 to 4 were each injected with 1mg/ml, 2mg/ml and 4mg/ml of hibiscus extract. Each sample was injected with volume of 200μl and the body weight was measure twice a week.

After 5 weeks of experiment, not a single mouse was died and the change of body weight was not significantly different with control group (Figure 8) and there was no striking morphological change either. Infer from this data, hibiscus extract showed no cytotoxicity against normal cells not only in vitro but also in vivo.

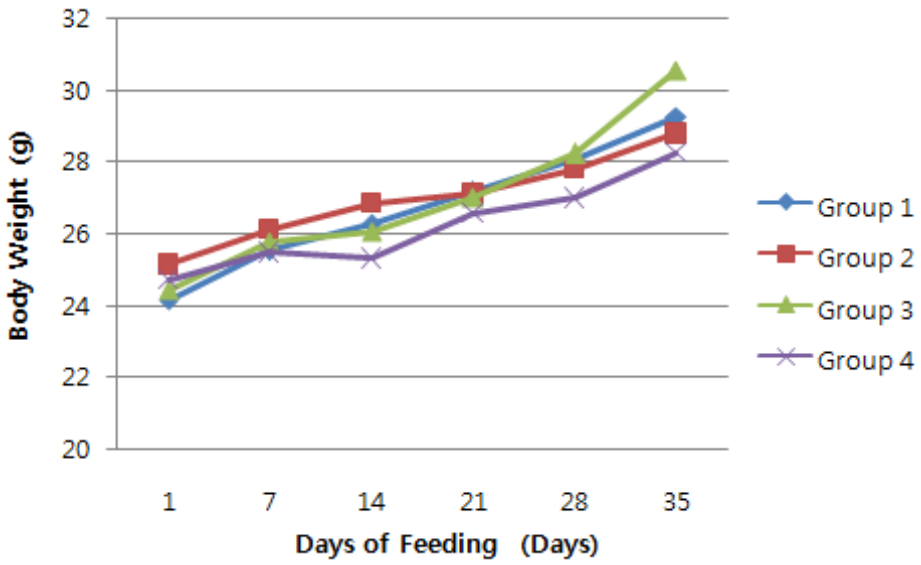


Figure 8. The effect of hibiscus exract on body weight changes. Group 1 was control group that was treated with sterile PBS and Group 2, 3 and 4 were test groups that were treated with each 1mg/ml, 2mg/ml and 4mg/ml of hibiscus extract. The amount of injection was 200μl per individual. The body weight progression of each group did not show significant differences.

3. Apoptosis assay

3.1. DNA fragmentation induced by HME.

To determine whether the hibiscus extract is able to induce apoptosis, colorimetric TUNEL assay was used to stain fragmented DNA. The U937 cells were exposed to 2 mg/ml of HME for 0-24 hours and 3'-OH termini were stained with Horse radish peroxide (HRP). DNA fragmentation is hallmark of apoptosis and the dye binds to free ends of DNA that are formed during fragmentation of DNA and stain cells with dark brown color.

The assay revealed that after treatment of hibiscus extract for 6, 12 and 24 hours, respectively, nubers of TUNEL-positive apoptotic cells were markedly increased (Figure 9).

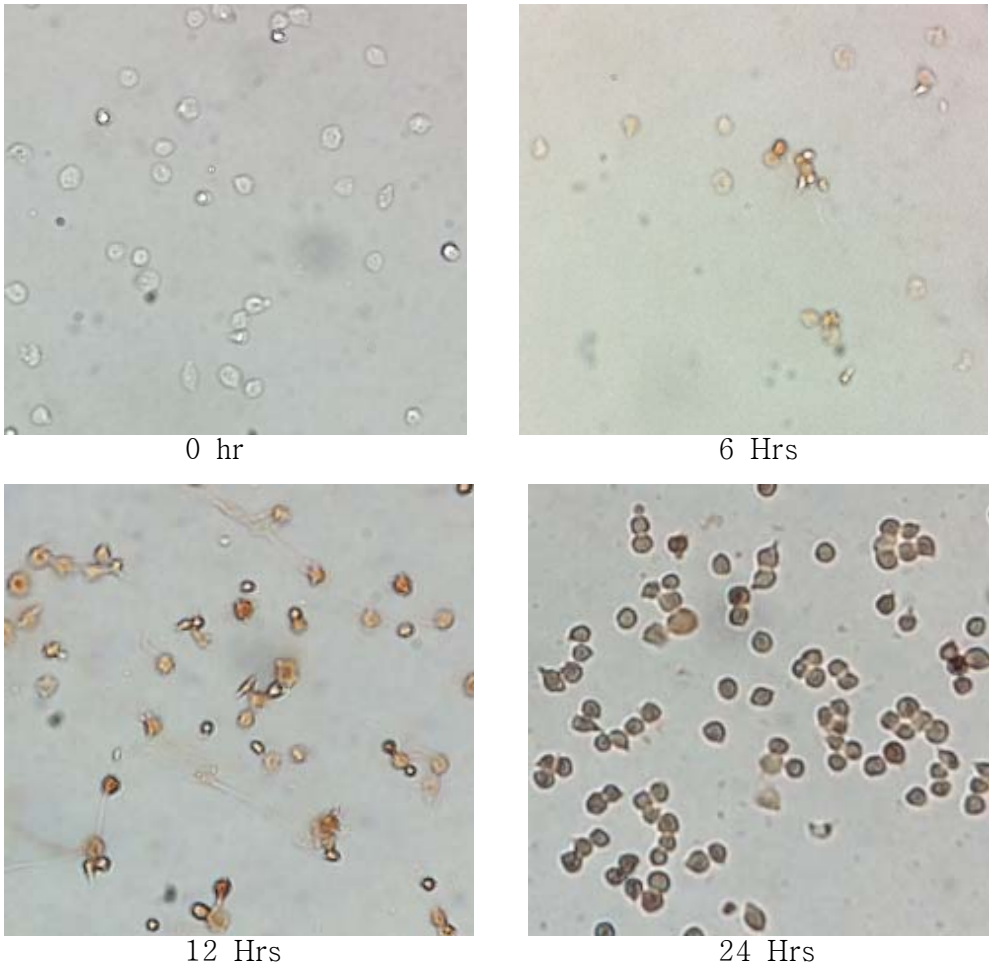


Figure 9. Hibiscus induces cell apoptosis by DNA fragmentation. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 hours, and DNA fragmentation was tested by DeadEnd tunel assay (Promega, USA).

3.2. HME induces mitochondrial membrane potential (ΔΨm) depolarization.

Mitochondria plays essential role in the maintenance of cell function and viability thus the mitochondrial membrane potential (ΔΨm) is important parameter of intrinsic death pathway. A decrease in mitochondrial membrane potential is an early universal event of apoptosis. Along with the membrane potential disruption, the mitochondrial membrane permeability allows the release of apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) to cytosol and activate apoptotic cascade.

For the experiment, the concentration of 2mg/ml was selected as an effective dose. The result indicated that the level of mitochondrial membrane potential was decreasing through the course of time and reached to the level of positive control by the time of 24 hours (Figure 10).

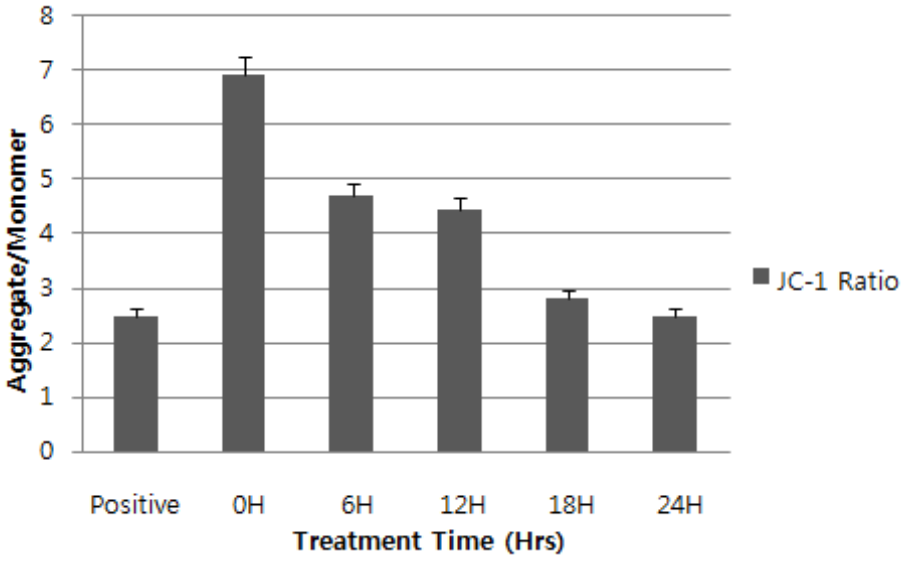


Figure 10. Hibiscus induces mitochondrial membrane potential (ΔΨm) depolarization. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 hours and mitochondrial membrane potential was measuered by ratio of aggregate/monomer using JC-1 dye. Values are mean ± S.E.M.

3.3. Activity of extrinsic apoptotic molecules, FasR and FADD

The Fas receptor (FasR) is a death receptor on the surface of cell that forms the death-inducing signaling complex upon ligand binding and induce extrinsic death pathway. Fas-associated protein with death domain (FADD) is an adoptor that binds to death domain of the death receptor and bridges the apoptotic signaling. In order to confirm whether HME-induced apoptosis involves in extrinsic pathway, the protein expression of FasR (Apo-1) and FADD were evaluated. The data showed complete loss of FasR protein expression while the constituent level of FADD did not change (Figure 15).

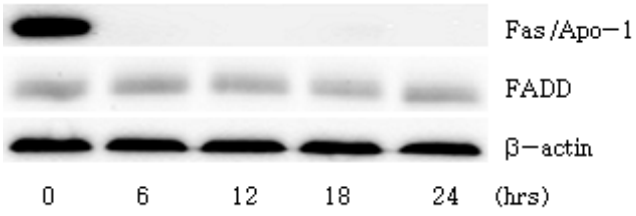


Figure 11. The effect of HME on Fas and FADD. U937 cells ($1x10^6$ cells/ml) were treated with HME for indicated hours then collected to measure the level of Fas and FADD by Western blot. Change in protein levels was observed in time dependent way.
The band intensity was measured by BIO1D

4. HME induces cell death in caspase independent way

Classically, the activation of caspase 3 by proteolytic cleavage is thought to be essential in cell apoptosis. It it responsible for the proteolytic cleavage of many key proteins. In recent findings, however, showed that apoptosis can occur in complete absence of caspases 3 activation. In order to measure activity of caspase 3, an executioner caspase, ELISA was performed as previously described. In brief, U937 cells were treated with 2mg/ml of HME for 0 to 24 hours and the activity was measured by amount of caspase cleavage. For comparison, the apoptosis-induced (positive) control was prepared by treating 50ng/ml of anti-Fas mAb for 24 hours. Also for inhibited apoptosis sample, z-vad-fmk, the caspase 3 inhibitor, was added to the cell one hour previous to adding anti-Fas mAb. The result demonstrated that there was no sign of caspase 3 activity through course of treatment time (Figure 11). This result infers that the cell death in U937 cells by hibiscus extract is independent from activation of caspase 3.

The inactivation of caspase 3 was double checked with western blot. As mentioned earlier, activation of caspase 3 requires cleavage processing of inactive zymogen into active p17 and p12 fragments. The antibody used for this experiment detects endogenous levels of full length caspase-3 (35 kDa) and the large fragment of caspase-3 resulting from cleavage (17 kDa). The result confirmed that there was no change in amount of full length caspase 3 nor increase of cleaved caspase fragments (Figure 12).

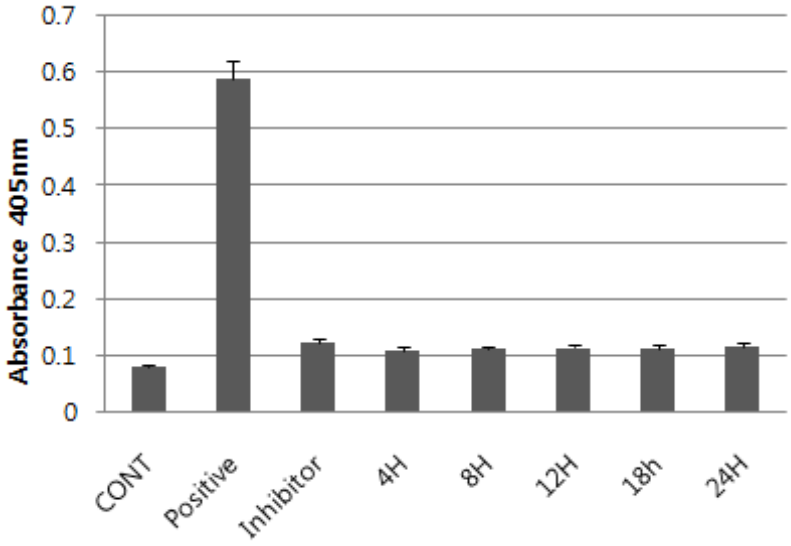


Figure 12. The effect of HME on caspase 3 activation. U937 cells were treated with HME for various hours then collected to measure the level of caspase 3 activity by Sandwich ELISA. The result is average of experiment replication for three times. Values are mean ± S.E.M (Positive; apoptosis induced with anti-Fas antibody. Inhibitor; Anti-Fas + z-vad-fmk (caspase inhibitor)).

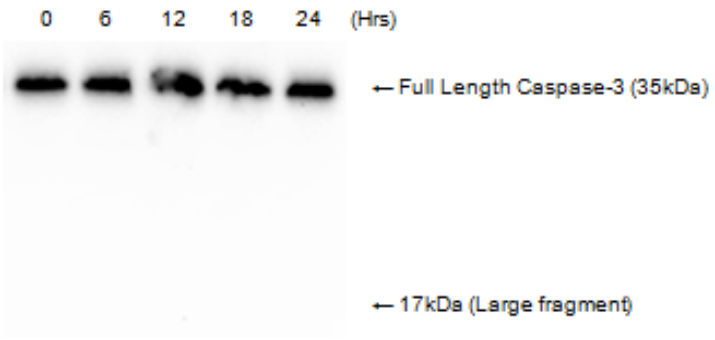


Figure 13. The effect of HME on caspase 3 cleavage. U937 cells were treated with HME for indicated hours then collected to measure the level of caspase 3 by Western blot. There was no cleavage observed

Lastly, to find out whether caspase inhibitor can affect the viablity of HME treated U937 cells, various concentrations of z-vad-fmk (caspase inhibitor) were pre-incubated with cells for one hour then different concentrations of hibiscus extract were treated to cell and incubated for 24 hours. Addition of z-vad-fmk was not able to inhibit cell death of U937 cell induced by hibiscus extract. Also there was no distinct change of cell viability between different concentration of z-vad-fmk (Figure 13).

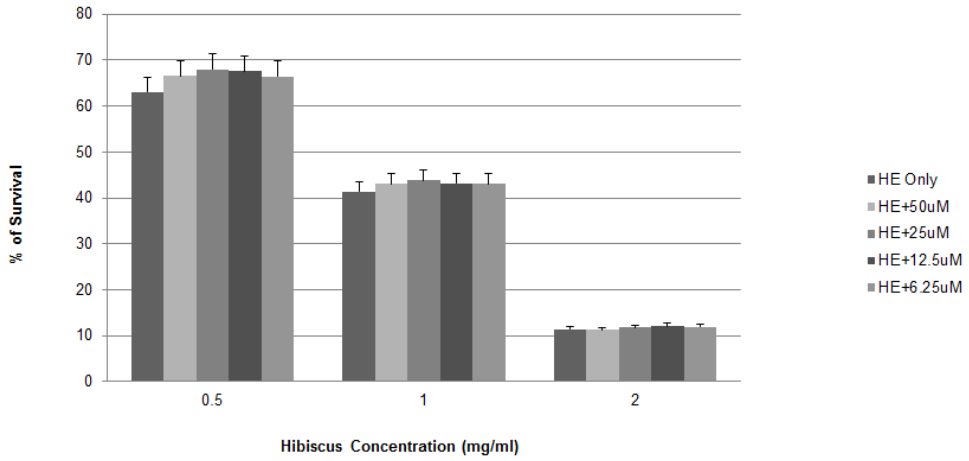


Figure 14. Caspase independent cell death induced by HME. U937 cells, seeded at density of $1x10^5$ cells/well, were treated with z-vad-fmk (caspase inhibitor) for one hour then various concentrations of HME were treated for 24hours. Cell viability was then measured by MTT assay. Values are mean ± S.E.M

5. Hibiscus extract induces apoptosis through mitochondrial pathway

5.1. HME mediates PARP cleavage on U937

As well as caspase cleavage, cleavage of poly ADP-ribose polymerase (PARP) is also an important marker for cell death. Normally, PARP is localized at nucleus and detects single-strand DNA damage and signals repair. Activated PARP is also known to have ability to induce apoptosis under stress. Similar with caspase, activation of PARP requires proteolytic cleavage and activated PARP can mediate the release of AIF from mitochondria via the production of PARP and this mechanism is caspase-independent process. Here, increase of cleaved PARP was observed in time dependent way. While the total amount of PARP was not change, the cleaved form of PARP showed significant increase (Figure 14).

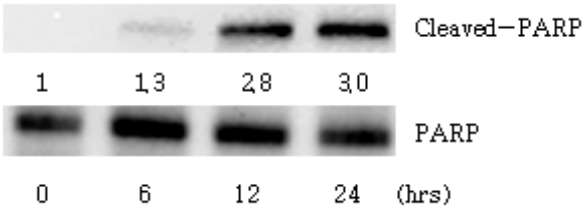


Figure 15. The effect of HME on PARP cleavage. U937 cells ($1x10^6$ cells/ml) were treated with HME for indicated hours then collected to measure the level of cleaved and uncleaved PARP by Western blot. Increase of cleaved PARP was observed in time dependent way. The band intensity was measured by BIO1D

5.2. Activation of MAPK pathway in u937 cell stimulated by HME.

mitogen-activated protein kinase (MAPK) family members can mediate survival of cell through complex kinase cascades and activated by different stress stimuli. The effect of hibiscus extract on three subfamilies of MAP kinases, extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38-MAPKs, were examined with western blot assay. First, the protein levels of ERK and phospho-ERK was examined. HME-treated U937 cells showed increased protein level of phosphotylated form of ERKs while the total ERK level stayed same (Figure 18). Unlike with ERKs, the two stress activated MAP kinases JNK and p38 MAPK were upregulated by hibiscus extract. The treatment of hibiscus extract seemed to affect only in active phosphorylated forms but did not changed the total protein level. The phosphorylated form of p38 and its upstream kinase molecule MKK3/6 were increased during the course of time (Figure 16). The other proapoptotic MAP kinase JNK was showed increased phosphorylation as well (Figure 17). Both phospho-p38 and phospho-JNK increased significantly after about 18 hours of treatment and their activation peaks almost synchronized. Yet, there was a notable apprearance of phosphorylated form of p38 MAPK at 12 hours, little earlier than p-JNKs, and this is probably due to activation of p-MKK3/6. The data showed that the level of p-MKK3/6 started to increase from 6 hours of treatment and peaks at 12 hour time period and the level of p-MKK3/6 decreased slowly. Thus the level of p-38 is probably affected by both activated JNKs and MKK3/6.

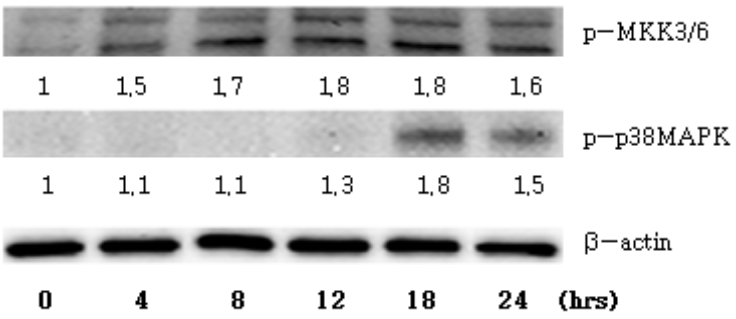


Figure 16. Treatement of HME increased p-MKK3/6 and p-p38. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 h, and whole-cell lysate was used for Western blotting analysis with p-MKK3/6 and p-p38MAPK antibodies. The band intensity was measured by BIO1D.

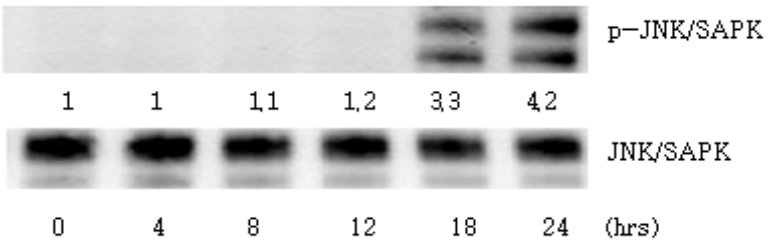


Figure 17. The effect of HME on JNK/SAPK and p-JNK/SAPK. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 h, and whole-cell lysate was used for Western blotting analysis with JNK/SAPK and p-JNK/SAPK antibodies. The band intensity was measured by BIO1D.

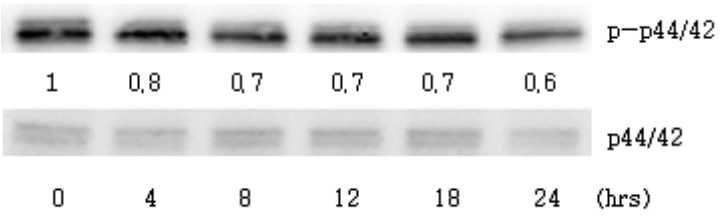


Figure 18. The effect of HME on p-ERK1/2 (p-44/42). U937 cells ($1x10^6$ cells/ml) were treated with HME for indicated time then collected to measure the level of p-44/42 by Western blot. The band intensity was measured by BIO1D.

5.3. HME mediates upregulation of PI3K/Akt pathway.

Since the previous data showed that treatment of hibiscus extract markedly attenuated the phosphorylated ERK, the expression of antiapoptotic proteins of PI3K/Akt pathway were also examined. 3'-phosphoinositide-dependent kinase-1 (PDK-1) is a serine/threonine protein kinase which phosphorylates Akt as an upstream kinase and regulates cell proliferation. The result showed that 2mg/ml of hibiscus extract was able to significantly inhibit both phosphorylated PDK1 and Akt (Figure 19).

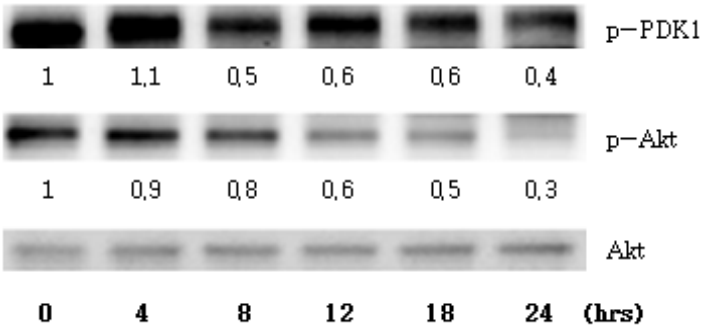


Figure 19. The effect of HME on PI3K/Akt pathway. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 hours, and whole cell lysates were used for Western blotting analysis with each p-PDK1, p-Akt and Akt antibodies. The band intensity was measured by BIO1D.

5.4. Attenuation of proapoptotic molecules in Bcl-2 family

As mentioned earlier, the mitochondrial death pathway is widely regulated by members of Bcl-2 family. In general, when the stress stimuli are received, the members of antiapoptotic Bcl-2 proteins, Bcl-2 and Mcl-1 are decreased while proapoptotic proteins, Bim, BAX and Bak are increasing. The level and activity of BH3-only protein, Bim is positively regulated by p38 MAPK and thus promote apoptosis. The mechanisms of which Bim induce apoptosis is via binding to antiapoptotic Bcl-2 and Mcl-1 proteins while mediating activation of proapoptotic proteins BAX, Bak and tBid. In nonapoptotic cells, Bcl-2 and Mcl-1 interrupt translocation of BAX and Bid to mitochondria and the level of Bim is attenuated. When apoptotic signal is received, the level of ERK and Akt is downregulated and increased p38 MAPK induces accumulation of Bim. Notably, increased Bim can induce apoptosis in dual mechanism by blocking Bcl-2/Mcl-1 and at the same time it can promote conformational change of BAX. Furthermore, the obvious accumulation of Bim was observed in time dependent fashion and the inactivated form of Bid was decreased as it goes though conformational change into active form (Figure 20).

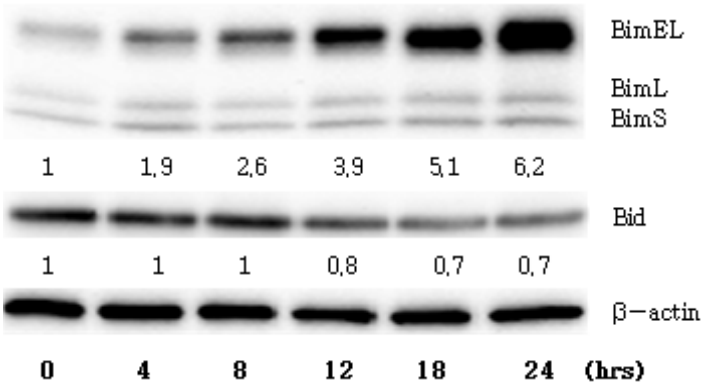


Figure 20. The effect of HME on antiapoptotic Bcl-2 family proteins, Bim and Bid. U937 cells ($1x10^6$ cells/ml) were treated with HME for indicated hours then collected to measure the level of Bim and Bid by Western blot. The band intensity was measured by BIO1D.

In addition, the protein expression of antiapoptotic Mcl-1 was also measured in time dependent manners. Mcl-1 is member of antiapoptotic Bcl-2 protein and many human leukemic cells constitutively overexpressing Mcl-1. It is known that Mcl-1 is required to prevent apoptosis during differentiation of U937 cells (Dale *et al.,* 2014). The protein level of Mcl-1 decreased at rather early in time course and it rapidly attenuated before 12 hour treatment (Figure 21).

BAX is primarily located in the cytosol in nonapoptotic cells but when apoptotic triggering stimuli are received, it undergoes conformational change and translocate into the mitochondrial membrane, promoting release of apoptotic factors such as cytochrome c and AIF. To evaluate the actual translocation of BAX, the mitochondrial and cytosolic fraction of HME treated U937 cells were analyzed separately. As a result, the protein level of cytosolic BAX was decreased as the mitochondrial BAX level built up (Figure 22).

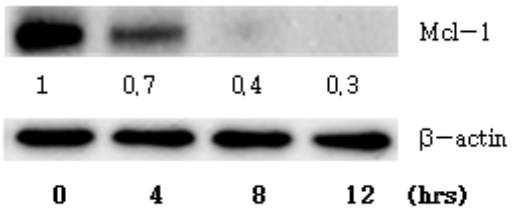


Figure 21. The effect of HME on antiapoptotic Mcl-1 protein expression. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-12 hours, and whole cell lysates were used for Western blotting analysis with Mcl-1 antibody. The band intensity was measured by BIO1D.

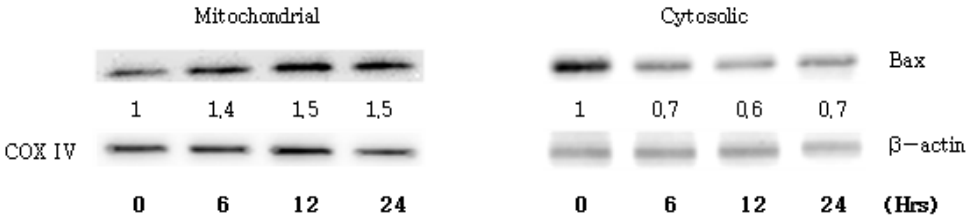


Figure 22. The effect of HME on BAX translocation. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 hours, and whole cell lysates were used for Western blotting analysis with each BAX antibody. The band intensity was measured by BIO1D.

5.5. HME mediates Cytochrome c and AIF release

Cytochrome c is small heme protein that is found in inner membrane of mitochondria and it is essential component of electron transport. However, apart from the role in ATP production, it is also the essential molecule in apoptosis. When cytochrome c is released from mitochondria, it promotes cell death via catalyzing assembly of apoptosome. The release of cytochrome c during HME-induced apoptosis was examined through course of time. The result indicated that cytochrome c was gradually decreased from mitochondrial fraction and increased in cytosolic cytochrome c level was increasing (Figure 23).

Another mitochondrial protein that is affected by translocation of BAX and Bid is apoptosis inducing factor, AIF. Different evidences from the literature indicate that the apoptotic inducing function of AIF is independent from caspase activity. Once released from mitochondria, AIF translocate into nucleus and mediate apoptosis. Although the mechanism of AIF is caspase independent AIF can also trigger the conventional mitochondrial death pathway and release of cytochrome c. In order to evaluate the release of AIF, the cytosolic level of AIF was measured and the data showed increased AIF level in cytosol occurred at 8 hours and reached at the maximal level at 12 hours. The level of AIF was then showed fluctuation (Figure 24).

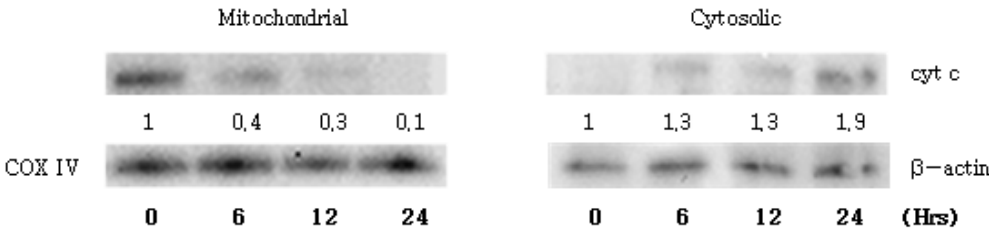


Figure 23. Treatement of HME induced translocation of cytochrome c. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 hours, and its cytosolic and mitochondrial fraction lysates were used for Western blotting analysis with cytochrome c antibody. The band intensity was measured by BIO1D.

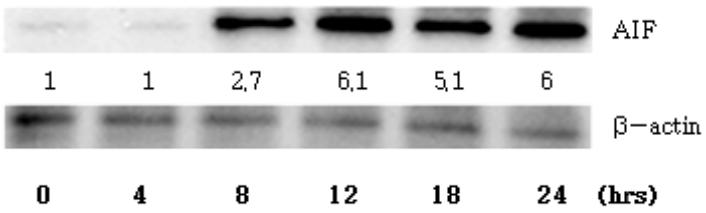


Figure 24. Treatement of HME induced translocation of AIF. U937 cells ($1x10^6$ cells/ml) were treated with 2mg/ml of HME for 0-24 hours, and cytosolic fraction of cell lysate was used for Western blotting analysis with AIF antibody. The band intensity was measured by BIO1D.

ChapterⅣ. Discussion

In the present study, we examined the anticancer potential of hibiscus extract on human leukemic U937 cells. *Hibiscus sabdariffa,* a natural polyphenol-rich plant that has been widely consumed in many regions as beverage or local medicines. Although there are various studies that proposed the anticancer ability of hibiscus, the exact mechanisms are still conflicting issue.

The calyces of hibiscus was extracted with various solvent to achieve its polyphenol compounds and amount of total polyphenols were evaluated (Figure 1). In this particular study, the methanolic extract was used to test whether hibiscus extract can induce cell death in U937 cell because it was one that exhibit the highest polyphenol contents. Prepared sample of extract was then subjected for analysis for the polyphenol components.

In order to test whether hibiscus extract can actually induce cell death, the selective cytotoxicity was examined in both normal human blood cells and various cancer cell lines including U937. The result showed that HME induced cell death in U937 cell as well as other cancer cells in time and concentration dependent manner but it didn't effect the survival of normal cell (Figure 4-6). Although the individual polyphenol found in hibiscus extract was known for its anticancer activity, the cytotoxicity of each polyphenol against U937 cell was not significant but there was some level of synergistic effect between quercetin and gallic acid (Figure 7). In addition, the noncytotoxic effect of hibiscus extract was tested in animal model as well (Figure 8).

HME treated U937 showed typical morphological and biochemical characteristics of apoptosis such as chromatin condensation, membrane blebbing, cell shrinkage, vacuolization, DNA fragmentation (Figure 9). However when the activation of caspase was measured,

there was no sign of cleavage. To make sure if the cell death was independent from caspase activation, the z-vad-fmk (caspase inhibitor) was treated to cell then HME was added in the cell. The result showed that cell death had been occurred independent from caspase activation (Figure 11-13). This finding was rather interesting because there was a paper about *hibiscus syriacus*, closely related subspecies with *hibiscus sabdariffa*, induced caspase independent apoptosis (Chang *et al.*, 2008).

Classically, it is known that caspase-independent apoptosis can occur through the release of apoptosis-inducing factor (AIF) from mitochondria (Hu *et al.*, 2013). Thus, release of AIF should be closely related to mitochondrial death pathway. Since there are many novel findings that MAPK pathway plays central role in intrinsic apoptosis of cell, the next target to look into was activation of this pathway. MAPK pathway is activated by different signals and with activation of p38, it regulates cell survival. The analysis of the activation of three MAPKs had been tested with western blot analysis. As a result, there was significant increase of phospho JNK and p38 MAPK while the level of phosphorylated ERK was attenuated (Figure 16-18). The mechanisms by which MAPK pathway can mediate survival of cell, include those involving the regulation of Bcl-2 family. The pro-apoptotic protein, Bim is suppressed by ERK/Akt and positively regulated by increase of p38MAPK. The level of Akt in HME-treated U937 showed gradual decrease in time dependent manner (Figure 19). On the other hand, there was a clear elevation of Bim while Bid was truncated in to active form (Figure 20). Accumulation of Bim caused downregulation of Mcl-1 and result in conformational change and translocation of BAX from cytosol to mitochondria (Figure 21-22). As a result, the

translocation of BAX caused release of cytochrome c and AIF (Figure 23-24) through mitochondrial dysfunction. Taken together, the present study explored the effect of hibiscus extract on complicated crosstalk between various intracellular pathways that alternatively induce apoptosis through caspase independent mitochondrial cell death. Therefore, this work would further expand the knowledge for the use of *hibiscus sabdariffa* as a potential anticancer agent.

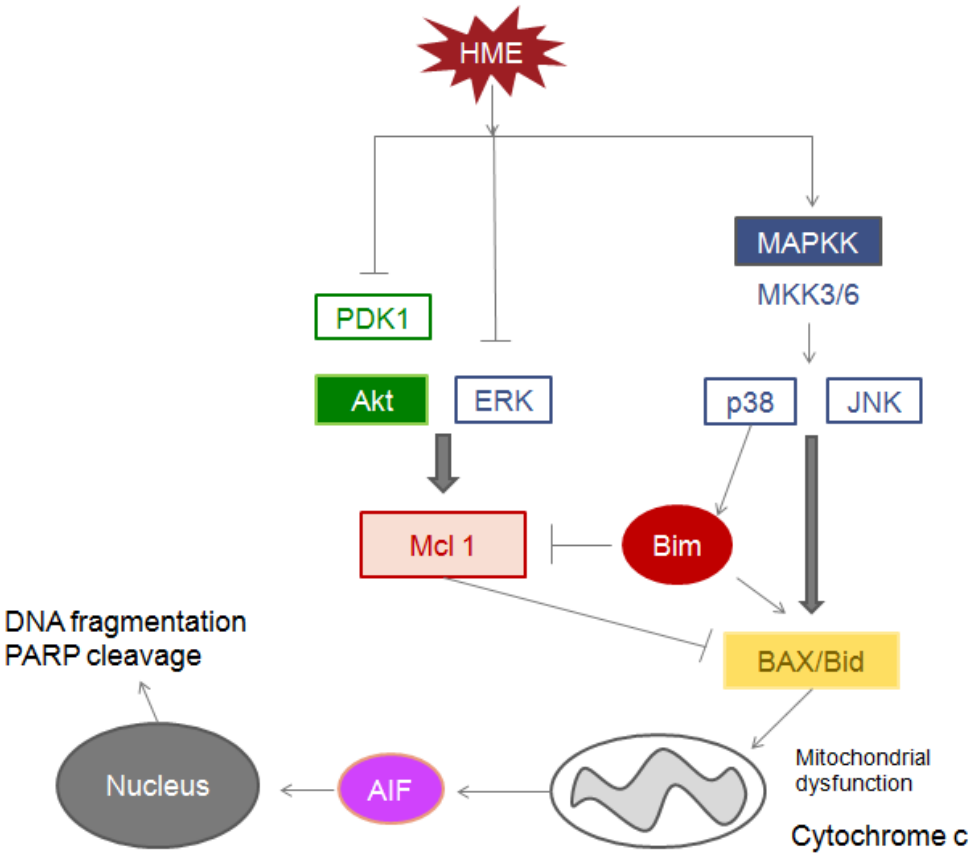


Figure 25. Proposed model of caspase independent mitochondrial death pathway induced by hibiscus methanolic extract on U937 cell.

Chapter Ⅴ. Reference

Bragado, P., Armesilla, A., Silva, A. and Porras, A. Apoptosis by cisplatin requires p53 mediated p38alpha MAPK activation through ROS generation. *Apoptosis*. **12(9),** 1733-1742.

C.J. Wang, J.M. Wang, W.L. Lin, C.Y. Chu, F.P. Chou, T.H. Tseng. (2000). Protective effect of Hibiscus anthocyanins against *tert*butyl hydroperoxide-induced hepatic toxicity in rats, *Food Chem.*

Cai B. and Xia Z. (2008). p38 MAP kinase mediates arsenite-induced apoptosis through FOXO3a activation and induction of Bim transcription. *Apoptosis.* **13(6)**, 803-810.

Chang H. C., Peng C. H., Yeh D. M., Kao E. S. and Wang C. J. (2014). Hibiscus sabdariffa extract inhibits obesity and fat accumulation, and improves liver steatosis in humans. *Food Funct.Toxicol.* **38,** 411-416..

Chang, Y. C., Huang, H. P., Hsu, J. D., Yang, S. F. and Wang, C. J. (2005). Hibiscus anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. *Toxicol. Appl. Pharmacol.* **205(3)**,201-12.

Chang, Y. C., Huang, H. P., Hsu, J. D., Yang, S. F. and Wang, C. J. (2005). Hibiscus anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. *Toxicol. Appl. Pharmacol.* **205**, 201-212.

Christiane Lakenbrink, Svenja Lapczynski, Beate Maiwald, and Ulrich H. Engelhardt. (2000). Flavonoids and Other Polyphenols in

Consumer Brews of Tea and Other Caffeinated Beverages *J. Agric. Food Chem.* **(48)**, 2848-2852

Cuenda, A. & Rousseau, S. (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. *Biochim. Biophys. Acta* **1773**, 1358-1375.

Cuevas, B. D., Abell, A. N. & Johnson, G. L. (2007). Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene* **26**, 3159-3171.

Datta, S.R. et al. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell*, **91(2)**, 231-241.

Dong S, Kang S, Gu TL, et al. (2007). 14-3-3 Integrates prosurvival signals mediated by the AKT and MAPK pathways in ZNF198-FGFR1-transformed hematopoietic cells. *Blood.* **110**. 360-369.

Fullerton, M., Khatiwada, J., Johnson J. U., Davis S. and Williams L. L. (2011). Determination of antimicrobial activity of sorrel (Hibiscus sabdariffa) on Escherichia coli O157:H7 isolated from food, veterinary, and clinical samples. *J. Med. Food*. **14(9)**, 950-956.

Ge, B., Xiong, X., Jing, Q., Mosley, J.L., Filose, A., Bian, D., Huang, S., Han, J., (2003). TAB1beta (transforming growth factor-beta-activated protein kinase 1 binding protein 1 beta), a novel splicing variant of TAB1 that interacts with p38alpha but not

TAK1. *Journal of Biological Chemistry* **278,** 2286-2293.

Herr I. and Debatin K.M. (2001). Cellular stress response and apoptosis in cancer therapy. *Blood*. **98(9),** 2603-2614.

Hou, DX, Tong, X, Terahara, N, Luo, D and Fujii, M. (2005). Delphinidin 3-sambubioside, a *Hibiscus* anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. *Archives of Biochemistry and Biophysics.* **440**, 101-109

Hu W., Ge Y., Ojcius D. M., Sun D., Dong H., Yang X. F. and Yan J. (2013). p53 signalling controls cell cycle arrest and caspase-independent apoptosis in macrophages infected with pathogenic Leptospira species. *Cell Microbiol*. **15(10)**, 1642-1659.

Kim B. J., Ryu S. W. and Song B. J. (2006). JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells. *J. Biol. Chem.* **281(30)**, 21256-21265.

Lakenbrink, C., Lapczynski, S., Maiwald, B., and Engelhardt U.H. (2000) Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. *Journal of Agricultural and Food Chemistry*, **48(7)**, 2848-2852,

Lecour S1, Lamont KT. (2011). Natural polyphenols and cardioprotection. *Mini Rev Med Chem.* **11(14),** 1191-9

Lin, HH, Huang, HP, Huang, CC, Chen, JH, and Wang, CJ. (2005) Hibiscus polyphenol-rich extract induces apoptosis in human gastric carcinoma cells via p53 phosphorylation and p38 MAPK/FasL cascade pathway. *Molecular Carcinogenesis.* **43(2)**, 86-99,

Manach, C., Williamson, G., Morand, C., Scalbert, A. and Remesy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **81**, 230S-242S.

Mittelstadt, P. R., Salvador, J. M., Fornace, A. J. Jr and Ashwell, J. D. (2005). Activating p38 MAPK: new tricks for an old kinase. *Cell Cycle* **4**, 1189-1192.

Papadopoulou, A. and Frazier, R.A. (2004) Characterization of protein-polyphenol interactions. Trends in food science & technology, **15 (3-4)**. pp. 186-190.

Perdiguero, E. et al. (2007). Genetic analysis of p38 MAP kinases in myogenesis: fundamental role of p38α in abrogating myoblast proliferation. *EMBO J.* **26**, 1245-1256.

Porras A, Zuluaga S, Black E, Valladares A, Alvarez AM, Ambrosino C, Benito M and Nebreda AR. (2004). *Mol Biol Cell*, **15**, 922-933.

Puthalakath H., O'Reilly L. A., Gunn P., Lee L., Kelly P. N., Huntington N. D., Hughes P. D., Michalak E. M., McKimm-Breschkin J., Motoyama N., Gotoh T., Akira S., Bouillet P.

and Strasser A. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. *Cell.* **129(7),** 1337-1349.

Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B. and Davis, R. J. (1996). MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* **16(3),** 1247-1255

Saeki, K., Kobayashi, N., Inazawa, Y., Zhang, H., Nishitoh, H., Ichijo, H., Saeki, K., Isemura, M. and Yuo, A. (2002). Oxidation-triggered c-Jun N-terminal kinase (JNK) and p38 mitogenactivated protein (MAP) kinase pathways for apoptosis in human leukaemia cells stimulated by epigallocatechin-3-gallate (EGCG): a distinct pathway from those of chemically induced and receptor-mediated apoptosis. B*iochem. J.* **368**, 705-720.

Spagnuolo, C,, Cerella, C,, Russo, M,, Chateauvieux, S., Diederich, GL. and Russo, M. (2011). Quercetin downregulates Mcl-1 by acting on mRNA stability and protein degradation, *British Journal of Cancer*. **105,** 221-230.

Stepniak, E. et al. (2006). c-Jun/AP-1 controls liver regeneration by repressing p53/p21 and p38 MAPKactivity. *Genes Dev.* **20**, 2306-2314.

Tsai, T.C., Huang, H.P., Chang, Y.C. and Wang, C.J. (2014). An Anthocyanin-Rich Extract from Hibiscus sabdariffa Linnaeus Inhibits N-Nitrosomethylurea-Induced Leukemia in Rats. *J. Agric. Food Chem.* **62**, 1572-1580.

Vela, L., Gonzalo, O., Naval, J. and Marzo, I. (2013). Direct Interaction of Bax and Bak Proteins with Bcl-2 Homology Domain 3 (BH3)-only Proteins in Living Cells Revealed by Fluorescence Complementation. *J. Biol. Chem.* **288(7)**, 4935-4946.

Zhang, L. N., Li, J. Y. and Xu W. (2013). A review of the role of Puma, Noxa and Bim in the tumorigenesis, therapy and drug resistance of chronic lymphocytic leukemia. *Cancer Gene Therapy.* **20**, 1-7

국문초록

히비스커스는 주로 동인도나 중국 등 열대지방에 분포해 자라고 있는 약용식물로써 학명은 *Hibiscus sabdariffa* Linnaeus 이다. 히비스커스의 꽃은 선명한 붉은빛을 띄고 있고 폴리페놀이 풍부하여 그 추출물은 항산화, 항비만, 항암 효과를 가진다고 알려져 있다. 또한 히비스커스의 꽃을 우려내 만든 차는 특유의 새콤한 맛으로 일상생활에서 음료로도 많이 섭취 되고 있다. 최근에는 히비스커스 추출물에서 발견되는 다양한 폴리페놀들이 여러 암세포에 대해 항암효과를 가지는 것이 확인되었는데 그 중 히비스커스 메탄올 추출물의 항암효과는 bio-assay를 통해 in vivo와 in vitro 모두에서 암세포에서만 apoptotic cell death 가 선택적으로 유도됨을 알 수 있었다. 그러나 히비스커스 추출물이 어떠한 기작을 통해 이러한 항암효과를 보이는지 혹은 어떠한 이유로 암세포에서만 선택적인 cell death를 일으키는지에 대해서는 아직 정확하게 알려져 있지 않다. 따라서 본 연구에서는 히비스커스의 추출물의 항암 기작 경로를 확인해 보았다.

먼저 히비스커스의 메탄올 추출물의 성분은 HPLC 분석을 통해 확인하였다. 폴리페놀의 성분과 함량은 Gallic acid (40.36%), caffeic acid (4.4%) 그리고 Quercetin (40.52%)로 밝혀졌다. 제일 먼저 히비스커스 추출물의 선택적인 항암작용을 보기 위하여 암세포와 정상세포 모두에 샘플을 농도별로 처리한 후 시간별로 세포의 viability를 측정한 결과 HME는 오직 암세포에서만 선택적으로 세포사멸을 유도하였다. 그 과정에서 DNA fragmentation과 mitochondrial dysfunction과 같은 형태적 생화학적 특징을 통해 세포의 apoptosis를 확인하였으나 기존의 cell apoptosis의 특징인 caspase 활성은 일어나지 않는 것으로 미루어보아 히비스커스에 의해 유도되는 세포사멸은 caspase-independent manner로 일어난다고 판단하였다. 또한 apoptosis의 과정에서 Fas등의 death receptor pathway molecule들은 관여하지 않는 것을 통해 apoptosis

mechanism은 오직 mitochondrial death pathway를 통해 일어나는 것을 확인하였다. 그 다음으로 HME에 의해 유도되는 apoptosis가 시간에 따라 어떠한 pathway의 expression에 관여하는지 알아보기 위해 세포에 HME를 처리 후 시간별로 각각의 pathway의 활성을 확인하였다. 그 결과 MAPK, PI3K/Akt, Bcl-2 families의 proapoptotic, antiapoptotic proteins의 activation을 본 논문을 통해 확인하였다. HME는 JNK, p38 MAPK, Bim등의 proapoptotic proteins은 축적시키고, antiapoptotic protein은 감소시키는 dual regulation을 통해 apoptosis를 유도한다. 그 후 이 signaling activation의 cascade는 mitochondria로부터 cytochrome c의 분비를 유도하고 이것은 다시 mitochondrial dysfunction을 수반하여 핵으로의 AIF translocation을 증가시킨다.

이러한 결과들을 종합하여, MAPK와 Akt pathway에 의해 유도되는 signaling cascade는 Bcl-2 family protein activation과 cytochrome c의 분비를 조절하여 mitochondrial membrane potential을 변화시켰다. 그 결과 AIF의 translocation은 human leukimic U937 cell에서 HME-induced caspase independent apoptosis에 중요한 역할을 하는 것으로 확인되었다.

주요어 : 히비스커스, 항암작용, 폴리페놀, Caspase-independent apoptosis, MAPK, Bcl-2, AIF
학번 : 2012-20313