The Beneficial Effects of Melinjo (*Gnetum gnemon* L.) Seed Extracts on Vasodilation and Cancer Cell Proliferation

(メリンジョ（*Gnetum gnemon* L.）種子抽出物が血管拡張および癌細胞増殖に及ぼす有益な効果**)**

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The Beneficial Effects of Melinjo (*Gnetum gnemon* L.) Seed Extracts on Vasodilation and Cancer Cell Proliferation

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for the degree

Doctor of Philosophy

by

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To my firstborn, Aiden

This one is for you

For all the walks in the parks without mommy to hold your hands

And all the naptimes without mommy to sing you lullaby…

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While my name is the only one written in front, I am by no means its sole contributor. For the past three years, there have been many people who have walked alongside me, guided me, helped me and made this journey possible. I would like to thank each and every one of them.

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CHAPTER I.

1.1. General Introduction

Throughout the ages, natural plants have been used to treat and help prevent several diseases (Wang et al., 2012). They provide unlimited opportunities for new drug discoveries due to the plethora of chemicals found in plant extracts (Achilonu and Umesiobi, 2015). The earliest written records about the medicinal uses of plants are dated from around 2600 BC, documenting the uses of around 1000 plant-derived substances in Mesopotamia (Cragg and Newman, 2013). These include oils of *Cedrus* species (cedar) and *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh), and *Papaver somniferum* (poppy juice). Every single one of them is still used today for the treatment of illnesses like cough and colds, parasitic infections and inflammation (Cragg and Newman, 2013). The best known record is the Ebers Papyrus, an Egyptian pharmaceutical record dating from 1500 BC, which documents approximately 700 plant-based drugs (Cragg and Newman, 2013).

The use of herbal medicines has been described throughout history in various forms such as traditional medicines, remedies, potions and oils. Even though the development and mass production of chemically synthesized drugs have transformed the global health care, large sections of the global population still rely on herbal medicine. This past decade, the use of herbal medicines or nutraceuticals has been widely accepted with many people now resorting to natural therapies and herbal remedies for treatment of various health challenges (WHO, 2004). Approximately four billion people living in developing countries depend on herbal medicinal products as their main source of healthcare and traditional medicinal practice, in which the use of herbs and plants are involved, is viewed as an integral

part of the culture in those communities (Ekor, 2013). On the other hand, the use of herbal medicinal products has increased immensely in developed countries during the past two decades. A survey on the usage of medicinal plants by the American public revealed an increase to over 37% in 1998 from a mere 3% in 1993 (Briskin, 2000). It was estimated that about 38% of adults and 12% of children were using some kind of traditional medicine in 2007 in the United States of America (Ernst et al., 2005). This shift to medicinal plants is commonly due to affordability, and agreement to public's ideology, which involves concerns on the harmful effects of synthetic medicines and the individualistic philosophy that encourages self-medication and personalized healthcare.

The protective effect and efficacy in treating diseases of plant-based medicine have been attributed to phytochemicals, the biologically active compounds found in plants. Previous researches revealed that phytochemicals are beneficial to health as: (1) substrates for biochemical reactions; (2) cofactors of enzymatic reactions; (3) inhibitors of enzymatic reactions; (4) absorbents/sequestrants that bind to and eliminate undesirable constituents in the intestine; (5) ligands that agonize or antagonize cell surface or intracellular receptors; (6) scavengers of reactive or toxic chemicals; (7) compounds that enhance the absorption and or stability of essential nutrients; (8) selective growth factors for beneficial gastrointestinal bacteria; (9) fermentation substrates for beneficial oral, gastric or intestinal bacteria; and (10) selective inhibitors of deleterious intestinal bacteria (Dillard and German, 2000). In addition, countless studies support that phytochemicals have beneficial roles in fighting against various diseases such as caners, coronary heart disease, diabetes, high blood pressure, inflammation, microbial, viral and parasitic infections,

psychotic diseases, spasmodic conditions, ulcers, etc. (Craig, 1997; Dillard and German, 2000; Achilonu and Umesiobi, 2015).

Phytochemicals are grouped according to the chemical nature of their putative active components. Among all phytochemicals, polyphenols are the most popular due to their properties and potential beneficial health effects. Polyphenols are a large group of phenylalanine derivatives that can be structurally characterized by the presence of at least two aromatic phenolic rings (C_6-OH) (Iriti, 2011). They are secondary metabolites of plants and are generally responsible for their defense against ultraviolet radiation or attack of pathogens (Pandey and Rizvi, 2009). Polyphenols are extensively studied for their antioxidant property. Antioxidant activity refers to the ability of polyphenols to prevent cell damage due to reactive oxygen species (ROS) or to prevent the generation of these species through iron binding (Perron and Brumaghim, 2009). Different ROS including superoxide, hydrogen peroxide, hydroxyl, and peroxyl radicals, are generated in the cells under normal and pathological conditions (Ataie et al., 2015). If ROS generation rate overcomes the ability of the antioxidant system of the cells, oxidative damage to DNA, proteins, and lipids will occur (Sun and Cheng, 1999). Oxidative damage to the DNA has been associated to be the cause of cancer; aging and neurodegenerative diseases such as Alzheimer's and Parkinson's disease; cardiovascular diseases such as arteriosclerosis; and is the primary cause of cell death and tissue damage resulting from heart attack and stroke (Perron and Brumaghim, 2009). Thus, oxidative stress prevention using antioxidants is very important in the treatment and prevention of diseases.

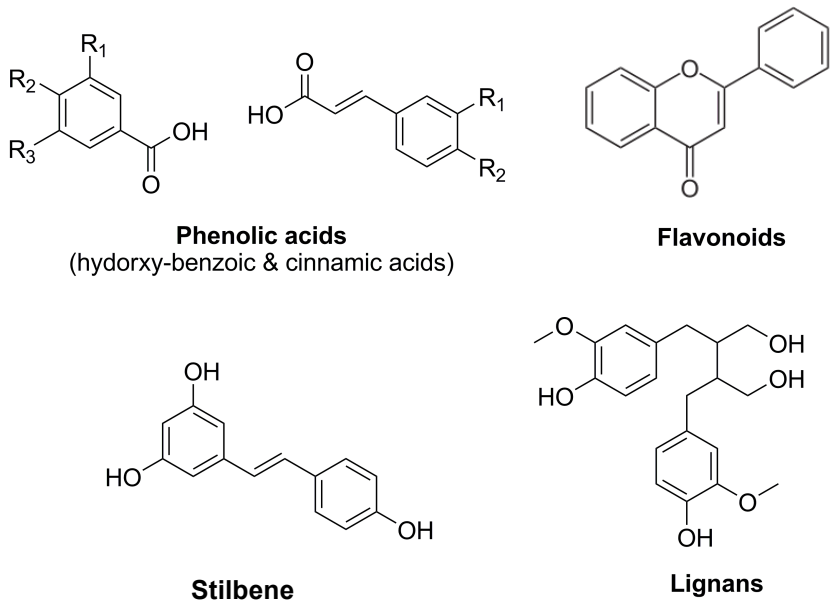

Fig. 1-1. Representative chemical structures of different classes of polyphenols.

Polyphenols are the most abundant source of antioxidants in human diet. Their total dietary intake can go as high as 1 g/d, which is definitely higher than that of all other classes of phytochemicals and other know dietary antioxidants (Scalbert et al., 2005). Polyphenols can be found in fruits, plant-derived beverages such as fruit juices, tea, coffee, and red wine, vegetables, cereals and dry legumes (Scalbert et al., 2005). Around 8000 polyphenolic compounds have been identified in different plant species (Pandey and Rizvi, 2009). Polyphenols can be classified into different groups according to the number of phenol rings that they have and the structural elements that link these rings to one another (Pandey and Rizvi, 2009). They are grouped in four main classes: phenolic acids, flavonoids, stilbenes, and lignans. Figure 1-1 illustrates the different groups of polyphenols and their representative chemical structures.

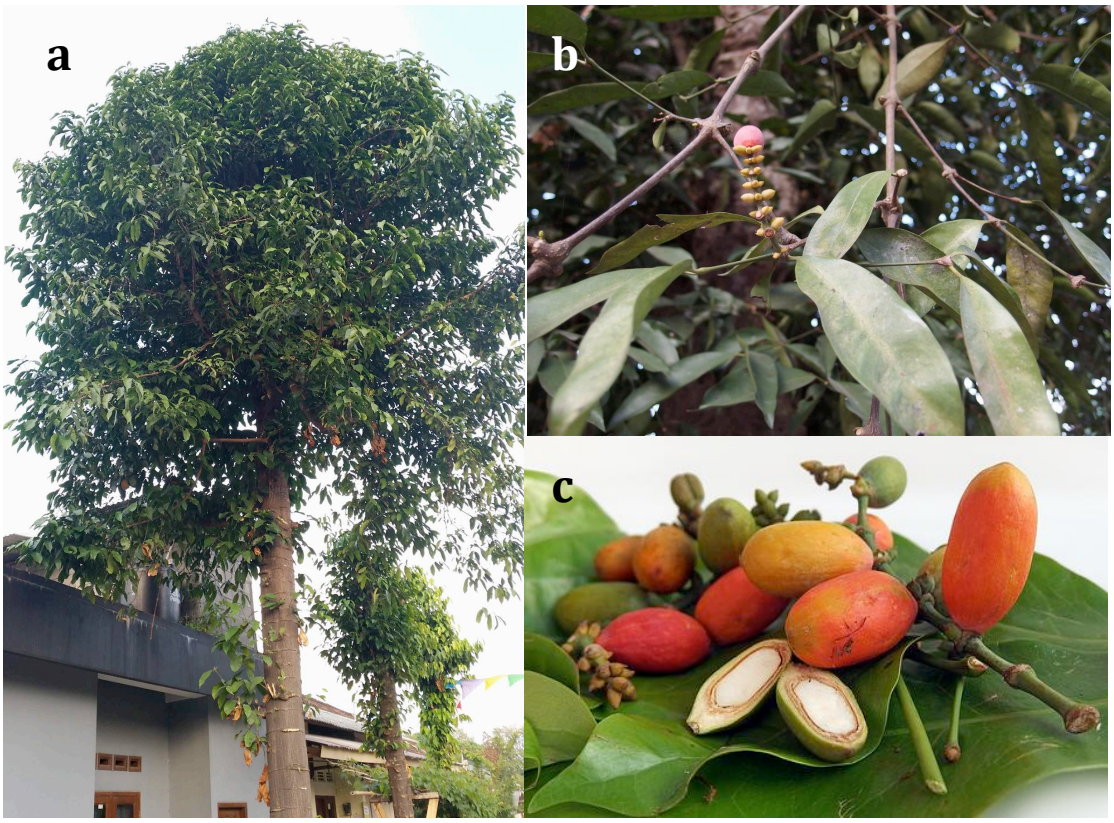

Fig. 1-2. Melinjo tree, leaves, fruits and its seed. (a) Melinjo tree taken from Indonesia. (b) Close up shot of melinjo leaves with a budding melinjo fruit. (c) Melinjo fruits in different levels of ripeness and a cross-section of the fruit showing the seed inside (photo from Indonesian Agency for Agricultural Research and Development)

Among all known polyphenols, one of the most studied is resveratrol (3,4′,5-trihydroxystilbene), a naturally occurring stilbene that is found largely in grapes, nuts and berries (Pandey and Rizvi, 2009). Numerous studies indicate the health benefits of resveratrol both *in vivo* and *in vitro*. Resveratrol has been found to exhibit antioxidant and anti-inflammatory effects on cardiovascular diseases, anti-diabetic, anti-obesity, anti-cancer bioactivities for both *in vitro* and *in vivo,* and anti-neurodegenerative bioactivity (Chun-Fu et al, 2013). Resveratrol exists in different isomeric forms and different forms exhibit different bioactivities. Resveratrol dimers are found abundantly in melinjo seeds. Melinjo (Indonesian name; *Gnetum gnemon*

L.*)* is an arboreal dioecious plant that is native in Southeast Asia but most abundantly found in Indonesia (Kato et al., 2009). It has a fruit that changes color from green, yellow, orange to red upon ripeness and inside the shell is its seed (Figure 1-2). It is popular in Indonesia that it is provided in their diet as soup and crackers. Ethanol extracts from the seeds of melinjo mainly contain gnemonoside A (resveratrol dimer, GCdiglucoside: 22% w/w), gnetin C (resveratrol dimer: 4.8% w/w), and gnemonoside D (resveratrol dimer, GC-monoglucoside: 6.9% w/w), with smaller amounts of trans-resveratrol (0.093% w/w) (Fig. 1-3) (Kato et al., 2009). Plants in this family are used in folkloric treatments for diabetes, arthritis, and bronchitis (Ota et al., 2009). Melinjo seeds have been reported to be safe for use as ingredients in functional foods or pharmaceuticals (Tatefuji et al., 2014). It was reported that oral administration of melinjo seed extracts (MSE) does not produce significant adverse effects to laboratory animals or humans (Narayanan et al., 2015), and oral administration of 1000 mg/ kg per day of melinjo seed extracts in rats had no observed adverse effect (Tatefuji et al., 2014). In fact, MSE is currently sold as a nutritional supplement in Japan (Narayanan et al., 2015).

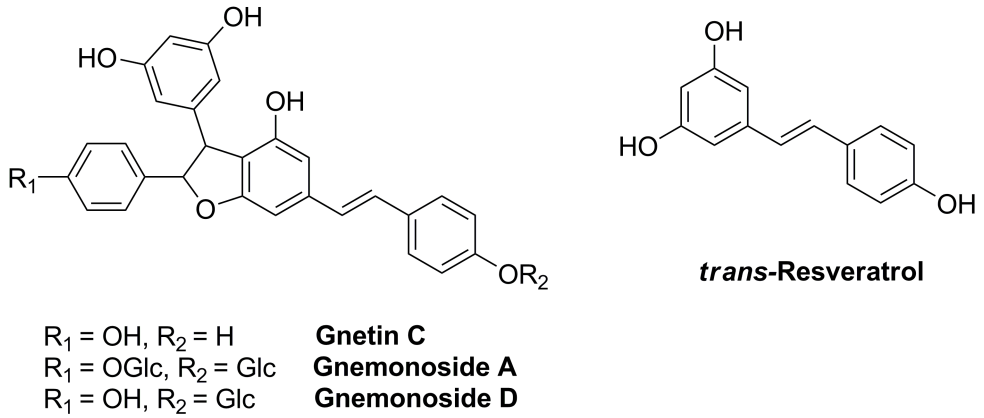

Fig. 1-3. Chemical structures of different stilbenes found in melinjo seed extracts.

Recent studies reported various pharmacological activities of MSE. Serum uric acid levels of non-obese Japanese males were decreased by MSE after 4 and 8 weeks of administration (Konno et al, 2013). Ethanol extracts of melinjo seeds showed radical scavenging effect, lipase and α-amylase inhibition activity, and antimicrobial activity against food microorganisms and enterobacteria (Kato et al, 2009). We have also reported that MSE intake during lactation stimulated hepatic AMP-activated protein kinase (AMPK) in the offspring of excessive fructose-fed pregnant rats (Kataoka et al, 2016). MSE treatment of high fat diet-fed mice improved survival and reduced the risk of death by 25%, indicating improvement of several aspects of metabolic syndrome (Ikuta et al, 2015). MSE also demonstrated angiotensin converting enzyme inhibitory activity (Mun'im et al, 2017). Gnetin C, a resveratrol dimer isolated from MSE showed inhibitory effects on tyrosinase activity and melanin biosynthesis (Yanagihara et al, 2012).

In addition, MSE suppressed multiple angiogenesis-related endothelial cell functions and/or tumor angiogenesis indicating its potential anticancer property (Kunimasa et al, 2011). MSE also showed antitumor activity against various human and murine tumor models *in vitro* (Narayanan et al, 2015). These results showed that MSE significantly inhibited the proliferation of pancreatic, prostate, breast and colon cancer cell types without affecting normal cells. This anti-tumor activity was validated *in vivo,* showing that 50 and 100 mg/kg per day of MSE administration significantly inhibited tumor growth, intratumoral angiogenesis, and liver metastases in BALB/c mice bearing colon-26 tumors (Narayanan et al, 2015).

Although various pharmacological activities of MSE have already been reported, much effort is still needed in order to further determine other beneficial effects of MSE. Specifically, there is still no comprehensive investigation on the mechanism by

which MSE causes cell death. In addition, *in vivo* studies using MSE are still limited and there is still no study on its effect in hypertension. Thus, the main objective of this study is to further explore the possible beneficial effects of MSE and their precise mechanism *in vivo* and *in vitro*. To attain such, first, *in vivo* experiment was performed to know the effects of MSE on the blood pressure levels and metabolism of offspring of pregnant rats that were fructose-fed during gestation. Then, *in vitro* studies were done to check the cytotoxicity of MSE against various cancer cell lines. To further investigate the anti-cancer potential of MSE, the mechanism on how MSE inhibited the proliferation of hepatocarcinoma cells (HepG2 cells) was determined. Figure 1-4 illustrates the schematic diagram for this study's research design.

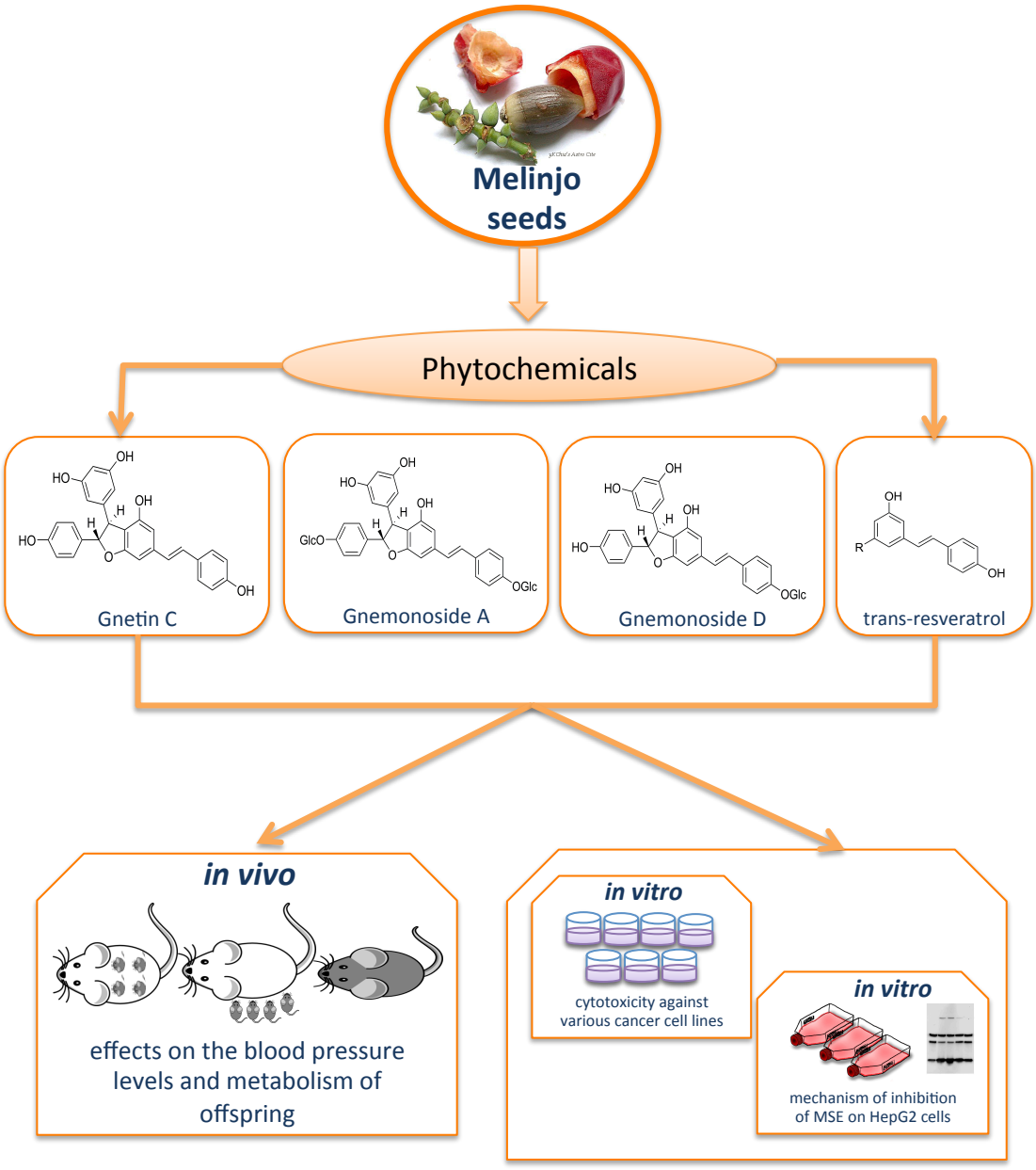

Figure 1-4. Schematic diagram of research design

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CHAPTER II.

Melinjo (*Gnetum gnemon* L.) Seed Extract Consumption During Lactation Improved Vasodilation and Attenuated the Development of Hypertension in Female Offspring of Fructose-fed Pregnant Rats

2.1. Abstract

Fructose intake has been correlated with increased prevalence of metabolic disorders including hypertension. In pregnant rats, fructose intake has been reported to have adverse effects on the health of its offspring. This study investigated the effects of gestational maternal fructose consumption and if supplementation with melinjo seed extracts to the maternal diet during lactation could benefit the offspring in later life. Pregnant rats were randomly divided into three groups: untreated (CC), fructose-treated (FC), and fructose and melinjo-treated (FM). FC and FM groups received 100 g/L of D(-)-fructose solution via the drinking water during gestation while CC received normal drinking water. During lactation, CC and FC groups were given standard commercial laboratory diet while FM group was given commercial laboratory diet with 0.1% melinjo seed extracts. After weaning, the offspring were given normal drinking water and standard commercial diet until week 17. The blood pressure of the offspring was monitored until the 16th week. During week 17, the offspring were sacrificed, and the kidneys were collected and analyzed. The level of renal phosphorylated AMP-activated protein kinase (pAMPK) in FM of 17-week female offspring was significantly higher compared to FC and CC groups. Maternal fructose intake down-regulated the renal endothelial isoform of NO synthetase (eNOS) expression in FC and maternal melinjo seed extract consumption maintained renal eNOS expression in FM of 17-week female offspring. In addition, maternal melinjo seed extract intake during lactation lowered the systolic blood pressure in FM of 17-week female offspring. Female offspring were

more vulnerable to the effects of placental fructose and melinjo seed extracts, suggesting sex-specific sensitivities. In summary, the data show that melinjo seed extract consumption during lactation improved vasodilation and attenuated the development of hypertension in the 17-week female offspring of fructose-fed pregnant rats.

2.2. Introduction

Organisms are vulnerable to environmental factors such as maternal nutrition during their early structural and physiological development. Maternal nutrition during pregnancy can permanently change an individual's postnatal phenotype thereby altering the offspring's susceptibility to diseases in later life (Vickers et al 2011). This concept of early nutritional or metabolic programming of adult health is supported by numerous experimental and epidemiological studies. One of the most significant is the study on Pima Indian children, which reports that the exposure to diabetes in utero during pregnancy contributed greatly to the development of childhood Type 2 Diabetes Mellitus, obesity, and hypertension, in addition to the risks that can be attributed to genetic factors alone (Dabelea et al 1998; Dabelea et al 2000; Charles et al 1994). Another notable research is about the effects of prenatal exposure to the Dutch famine on adult diseases in later life. Their findings showed that chronic diseases originate through adaptations made by the fetus in response to undernutrition. They suggested that risk factors for coronary heart disease have their origins in utero, but found to be programmed at different times. In addition, they claimed that maternal malnutrition during gestation may permanently affect adult health without affecting the size of the baby at birth (Roseboom et al 2001).

Over the past few decades, the occurrence of metabolic disorders such as obesity and Diabetes Mellitus has dramatically increased to epidemic proportions worldwide. This prevalence of such metabolic disorders was observed to coincide with the significant increase in fructose consumption. Fructose, a naturally occurring sugar found in many fruits and honey, became a staple additive in human diet over the past 40 years. This is due to the increased usage by manufacturing companies of high fructose-containing corn syrup, a cheaper alternative to sucrose. Studies on humans (Stanhope et al 2010; Tappy et al 2010) and animals (Basciano et al 2005; Le et al 2006) have demonstrated that overconsumption of fructose leads to the development of components of metabolic diseases, including insulin resistance, dyslipidemia, and hypertension, among others. During pregnancy, some researches revealed that gestational dietary fructose ingestion causes harmful changes in the development of fetuses. Maternal fructose intake during pregnancy of rats has been shown to upregulate the expression of maternal and fetal hepatic sterol regulatory element-binding protein-1c (Mukai et al 2013) and to affect maternal and fetal leptin signaling in their offspring (Rodriguez et al 2013). Another study reveals that fructose-induced diabetes during pregnancy could affect the development of sustained hypertension in the pregnant rats (Olatunji-Bello et al 2001).

Since fructose intake has been reported to cause hypertension during pregnancy, we hypothesized that administering fructose during gestation would also affect the development of the offspring. In addition, we suggested that supplementing bioactive food components to the mothers could offset these adverse consequences to the offspring exposed to dietary fructose during gestation. Resveratrol, a polyphenolic compound found in various plants and exists in different isomeric forms, is a bioactive food component reported to prevent hypertension and

reduce blood pressure (Dolinsky et al 2013; Milanovic et al 2012). Resveratrol dimers are abundant in melinjo (Gnetum gnemon) seeds. Ethanol extracts from the seeds of melinjo mainly contain gnemonoside A (resveratrol dimer, GCdiglucoside: 22% w/w), gnetin C (resveratrol dimer: 4.8% w/w), and gnemonoside D (resveratrol dimer, GC-monoglucoside: 6.9% w/w), with smaller amounts of trans-resveratrol (0.093% w/w) (Kato et al 2009). Melinjo seeds are part of the Indonesian cuisine due to their high nutritive value. Plants in this family are used in folkloric treatments for diabetes, arthritis, and bronchitis (Ota et al 2013). It was reported that oral administration of melinjo seed extracts (MSE) does not produce significant adverse effects to laboratory animals or humans (Narayanan et al 2015) and oral administration of 1000 mg/kg per day of MSE in rats had no observed adverse effect level (NOAEL) (Tatefuji et al 2014). Resveratrol dimers extracted from melinjo seeds were reported to have antiangiogenic effects, inhibitory effects against endothelial senescence, tyrosinase activity and melanin biosynthesis and cancer preventive effects (Ota et al 2013, Kunimasa et al 2011, Yanagihara et al 2012).

Although resveratrol has already been extensively studied, the health benefits of resveratrol dimers from MSE still need further research. Specifically, there is little knowledge on the effect of melinjo resveratrol dimers on the blood pressure levels and metabolism of offspring of pregnant rats that were fructose-fed during gestation. In this study, pregnant rats that were fructose fed during gestation period were fed with MSE during lactation period. The blood pressure of the offspring was monitored until the 16th week. In addition, kidney samples were dissected and western blotting was performed. This study aims to determine the effects of melinjo seed extracts on the metabolism and development of hypertension of the offspring of fructose treated pregnant rats.

2.3. Materials and Methods

2.3.1 Melinjo seed extraction

In September 2012, melinjo seeds were collected from Bogor, West Java, Indonesia. Dried melinjo seeds were homogenized and immersed in 50% ethanol solution at room temperature for one hour. The filtrate was collected after centrifugation for one hour at 2000 x g for 20 min at room temperature. It was then concentrated through rotary evaporation and freeze-dried. Folin-Ciocalteu method (Singleton et al 1965) was used to analyze the total polyphenol content in the melinjo extract. The total polyphenolic compounds in 100 grams melinjo extract were evaluated as equivalent of 9.3 g of (+)-catechin hydrate.

2.3.2 Characterization of melinjo seed extract

The powder of melinjo seeds (11.1 mg) was dissolved in 50% EtOH (2 mL) and soaked for an hour. This solution was filtered by using a 0.5 μm PTFE filter and was subjected to LC/MS analysis on a Bruker Daltonics micrOTOF-HS mass spectrometer (ESI) coupled with an Agilent 1100 Series HPLC system. The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 μm, 25 °C) under the following conditions: 0–25 min, gradient elution of 50–100% MeCN with 0.1% (v/v) formic acid in H_2O; 25–35 min, isocratic elution of 100% MeCN with 0.1% (v/v) formic acid in H_2O, flow rate: 0.2 mL/min. The % composition was calculated by integration of the peak area using the Compass 1.3 DataAnalysis software. Results showed that the seed extract contains gnemonoside D (~30%) and gnetin C (~20%).

2.3.3 Animal experiments

The performed animal study was approved by the Animal Research Committee, Aomori University of Health and Welfare, and all experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Aomori University of Health and Welfare (Permission number; 12006).

Seven-week-old male and virgin female Wistar rats were obtained from Clea Japan (Tokyo, Japan). The rats were housed under a 12 h light-dark cycle with constant temperature of 23 ± 1°C with food and tap water available ad libitum. Using a vaginal impedance reader (Model MK-10C; Muromachi Kikai, Osaka, Japan), female rats were determined to be in the appropriate stage of the estrous cycle for mating at 12-13 weeks of age. This was a routine procedure done in the afternoon and a reading of > 3kΩ indicated that the females were in pro-estrous and presumably in estrous. One female rat was mated with one male rat overnight. The following morning, a vaginal plug was observed indicating a successful mating, thus marked as Day 0 of gestation.

Experimental scheme is shown in Figure 2-1. Pregnant rats were randomly divided into three groups: untreated (CC; n = 4), fructose-treated (FC; n = 4), and fructose and melinjo-treated (FM; n=4). FC and FM groups received 100 g/L of D(-)-fructose solution (Wako Pure Chemical Industries, Osaka, Japan) via the drinking water during gestation. On gestational Day 21, dams were allowed to deliver spontaneously. Following delivery, the drinking water of FC and FM was exchanged for normal drinking water. CC and FC groups were given both drinking water and a standard commercial laboratory diet (MF Diet; Oriental Yeast, Tokyo, Japan) ad libitum throughout gestation and lactation. Conversely, FM group was given drinking

water and commercial laboratory diet with 0.1% melinjo seed extract. According to the manufacturer's description, the MF diet consisted of 7.7% moisture, 23.6% protein, 5.3% fat, 6.1% ash, 2.9% crude fibre and 54.4% nitrogen-free extract (including carbohydrates). A previous study reported that the production of the T-helper cytokine was enhanced in mice orally treated with a 50% ethanol extract of melinjo at 100 mg/kg/day, implicating that melinjo extract has a physiological function. From this report, a diet containing 0.1% MeE, which was equivalent to approximately 30–80 mg MeE/day, was used during the lactation period (Kataoka et al 2016). On week 3 (weaning stage), lactation period stops and the offspring were given normal drinking water and standard commercial laboratory diet until the week 17. Before sacrifice at week 17, the animals (male:CC, n=5; FC, n=5; FM, n=6 and female: CC, n=6; FC, n=5; FM, n=6) were weighted and fasted overnight. After the rats had been killed, the both kidney were removed immediately, washed in ice-cold saline, weighed and frozen in liquid nitrogen and stored at -80°C.

2.3.4 Systolic blood pressure (SBP) measurement

Before the measurements, rats were placed in a chamber prewarmed at 35 ° C for 10 min. Thereafter, the rat was fixed to the animal fix holder, and cuff pulse sensor was attached to the tail. When the pulse sound was heard rhythmically and the size of the pulse has stabilized, the pulse wave of the caudal artery was measured non-invasively under anesthesia by the tail-cuff plethysmography method (model MK-1100, Muromachi Kikai Co. Ltd, Tokyo, Japan). The SBP of rats was measured on 6th, 12th and 16th weeks. The final SBP were presented as the mean of 5 measurements. Experimental measurements were performed between 9:30 h and 13:00 h.

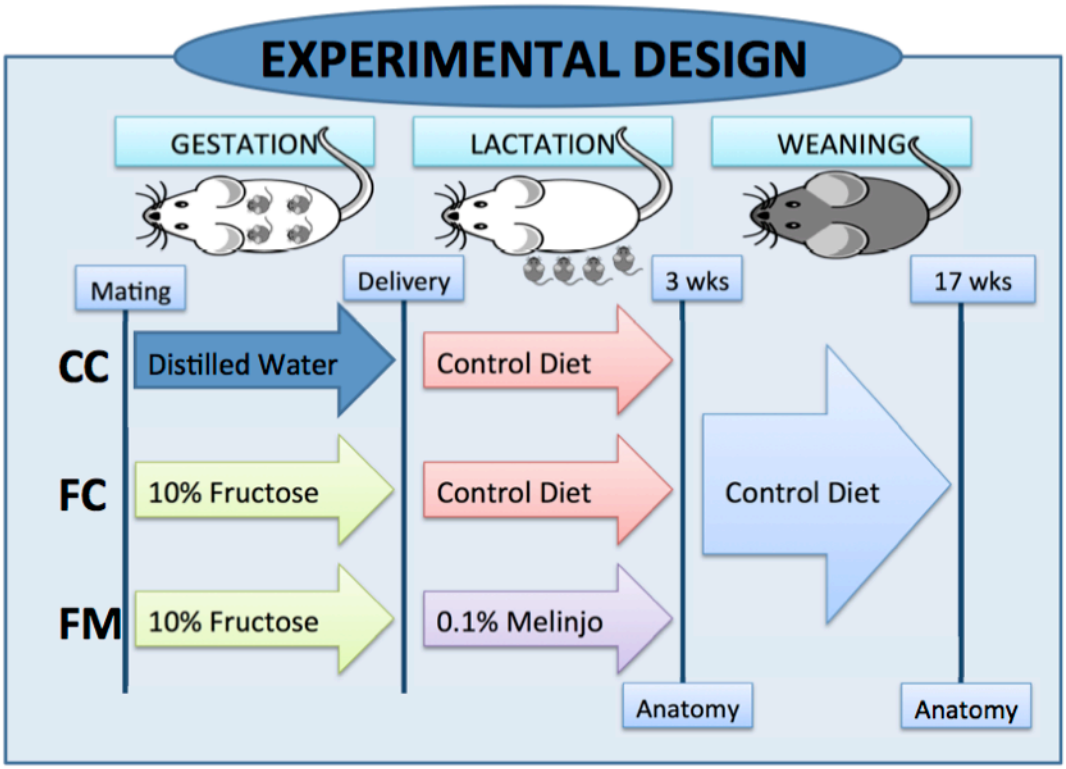

Figure 2-1. Schematic diagram of experimental design. Pregnant Wistar rats were fed control (distilled water) and high fructose (10% fructose) diets during gestation. During lactation, each dam received a control or 0.1% melinjo seed extract-containing control diet. During the weaning period, all offspring received control diet.

2.3.5 Western blotting

The kidneys were homogenized by adding homogenizing buffer (50 mM HEPES, 150 mM NaCl, 1 mM dithiotheitol and 0.5% (v/v) Tween-20; pH7.4) with protease inhibitor cocktail tablets (Roche Applied Science, Basel, Switzerland). After homogenization, centrifugation of the homogenates was done at 5000 × g for 45 min at 4°C. The supernatants were collected and the measurement of the protein concentration was done using BCA protein assay kit (Pierce, Rockford, IL, USA). Electrophoresis was performed using 10% sodium dodecyl sulphate-poly-acrylamide gel. The resolved proteins in the gel were transferred to polyvinylidene fluoride membranes (GE Healthcare UK Ltd., Buckinghamshire, UK). After transfer, the membranes were incubated with antibodies for AMP-activated protein Kinase α

(AMPKα), phosphor-AMPKα- Thr172 (pAMPKα) polyclonal antibody, and endothelial isoform of nitric oxide synthase (eNOS) antibody. The membranes were then washed followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies. The bands were visualized by chemiluminescence using ECL western blotting detection reagents (GE Healthcare UK). Quantitative analysis of the specific band density was performed using ATTO densitometry software (ATTO Corp., Tokyo, Japan). Protein levels were normalized to β-actin expression from the same sample.

2.3.6 Statistical analysis

Statistical analyses were performed using SPSS version 20.0.0 for Windows (IBM SPSS Statistics; IBM Corporation, Somers, NY, USA). Data were tested for maternal diet effect using the unpaired t-test. In order to determine lactation diet effect, analysis of variance (ANOVA) among experimental groups in the different experimental periods was used. The Tukey test for multiple comparisons was used to identify specific differences in the variables that met the criterion for statistical significance ($P < 0.05$). Data are expressed as the mean ± SE.

2.4 Results

2.4.1 Kidney weight and relative kidney weight of offspring

There was no significant difference in the body weights of both male and female offspring among the three groups in postnatal weeks 3 and 17 as previously described (Kataoka et al 2016). In addition, the kidney weights of male and female offspring did not differ significantly among the three groups for both postnatal weeks 3 and 17 (Table 2-1).

Table 2-1. Kidney weights at the time of sacrifice

Kidney weight (g)	male			female		
	CC	FC	FM	CC	FC	FM
3 weeks	0.50±0.02	0.48±0.03	0.50±0.02	0.47±0.02	0.46±0.03	0.51±0.02
17 weeks	2.58±0.08	2.58±0.07	2.64±0.13	1.53±0.04	1.60±0.09	1.59±0.13

Mean±SE (3 weeks: CC, n=10; FC, n=7; FM, n=10. 17 weeks: CC, n=6; FC, n=5; FM, n=5-6).

2.4.2 Expression and phosphorylation of AMPK and eNOS

The renal expression of AMPK in the male offspring at postnatal week 17 did not differ among the three groups (Figure 2-2A). In addition, the level of phosphorylation of AMPK in the FC group of the male offspring at postnatal week 17 showed some tendency to be higher than CC and FM (Figure 2-2B) but still, there was no any significant difference. Similarly, the total AMPK expression in the female offspring at postnatal week 17 did not show any difference among the three groups (Figure 2-3A). However, the level of pAMPK in the FM group was significantly higher compared to FC and CC groups (Figure 2-3B).

At postnatal week 17, the renal eNOS did not differ among the three groups for the male offspring (Figure 2-4A). However, for the female offspring, the level of eNOS in the FC group was significantly lower compared to CC (Figure 2-4B), while FM group was significantly higher compared to FC but not to CC (Figure 2-4B). This indicates that maternal fructose consumption down regulated eNOS expression in FC but this was countered by maternal melinjo seed extract intake during lactation period.

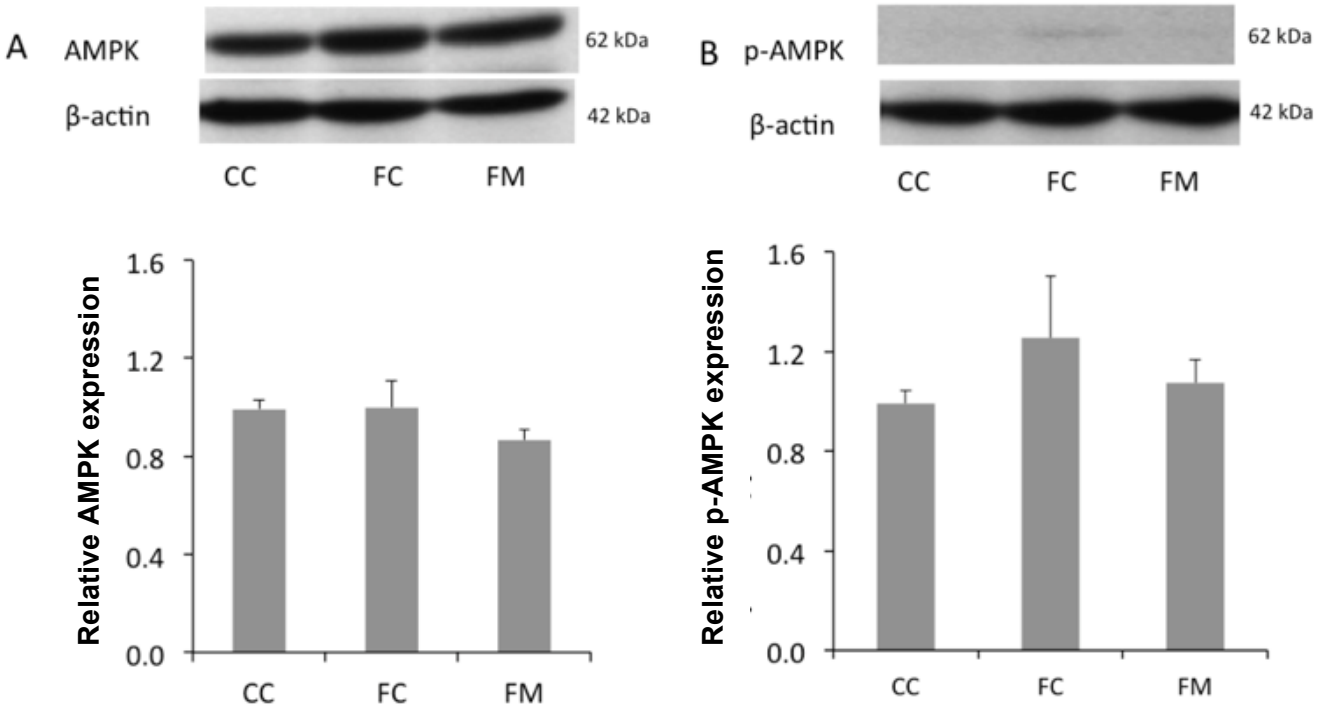

Figure 2-2. Contents of AMPK (A) and pAMPK (B) in kidney of male offspring at 17 weeks of age. Each bar shows mean ± SE (CC, n=6; FC, n=5; FM, n=6).

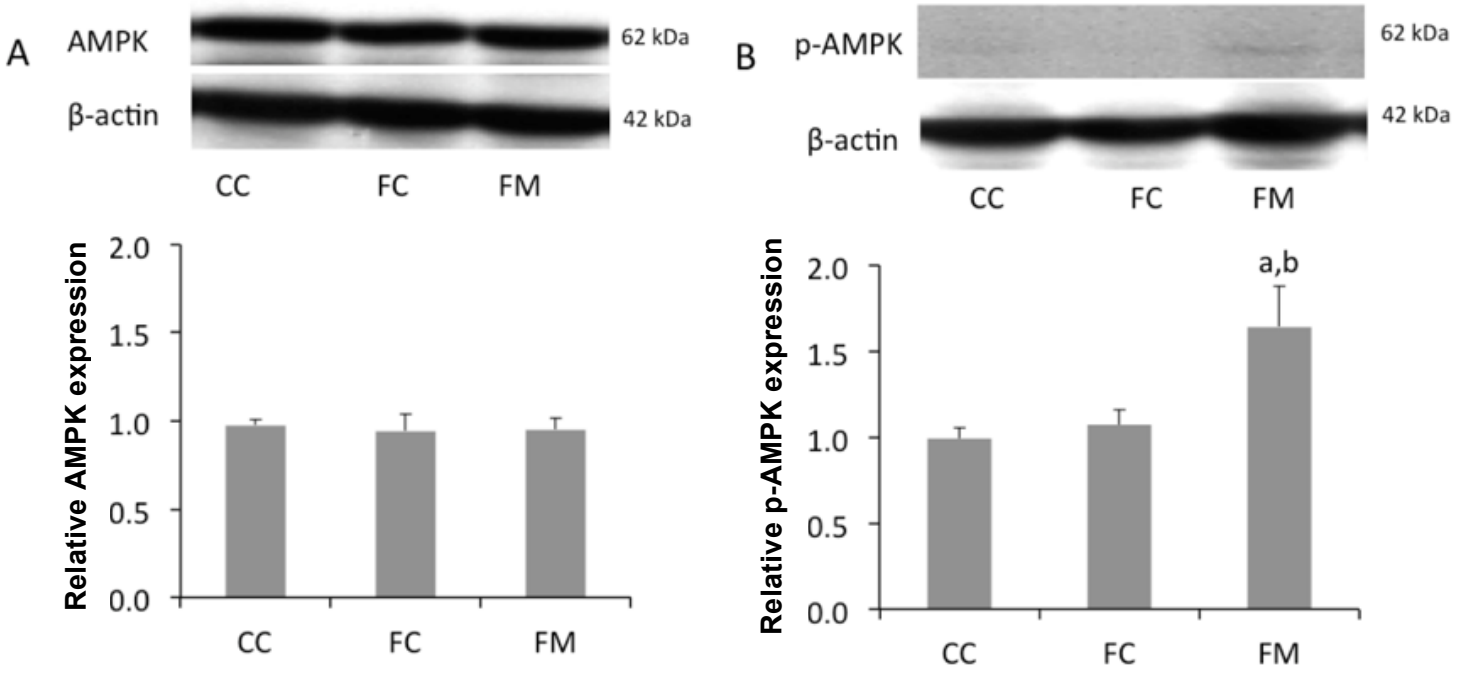

Figure 2-3. Contents of AMPK (A) and pAMPK (B) in kidney of female offspring at 17 weeks of age. Each bar shows mean ± SE (CC, n=6; FC, n=5; FM, n=5). a and b indicate $p < 0.05$ versus CC and $p < 0.05$ versus FC, respectively.

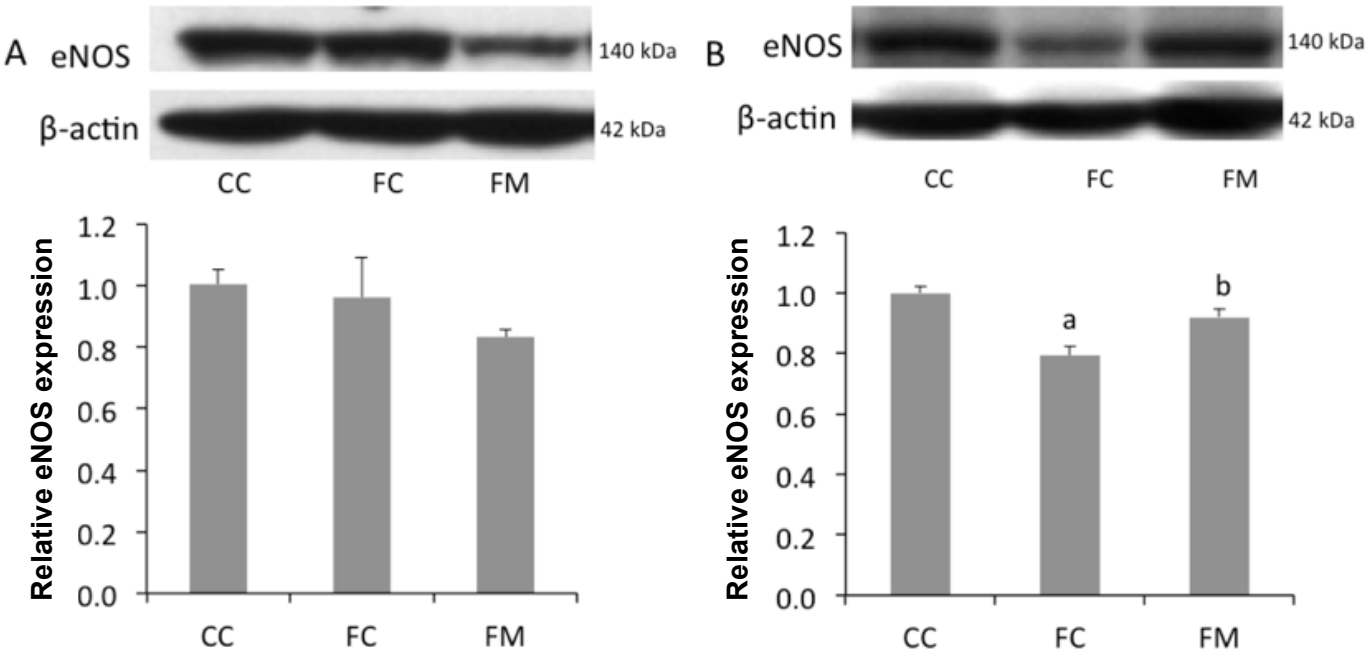

Figure 2-4. Contents of eNOS in kidney of male (A) and female (B) offspring at 17 weeks of age. Each bar shows mean±SE (in A: CC, n=6; FC, n=5; FM, n=6; and in B: CC, n=6; FC, n=5; FM, n=5). a and b indicate $p < 0.05$ versus CC and $p < 0.05$ versus FC, respectively.

2.4.3 Effects of melinjo intake in systolic and diastolic blood pressure

The SBP of the male offspring did not differ among the three groups all throughout the weaning period (Figure 2-5A). Conversely, the SBP of FC group of the female offspring at week 16 was significantly higher compared with CC while FM did not show any difference against the control (Figure 2-5B). This indicates that maternal melinjo seed extract intake during lactation prevents the rise in SBP, which was induced by maternal fructose consumption. The diastolic blood pressure (DBP) for both male and female offspring did not show any difference during the weaning period (Figure 2-6).

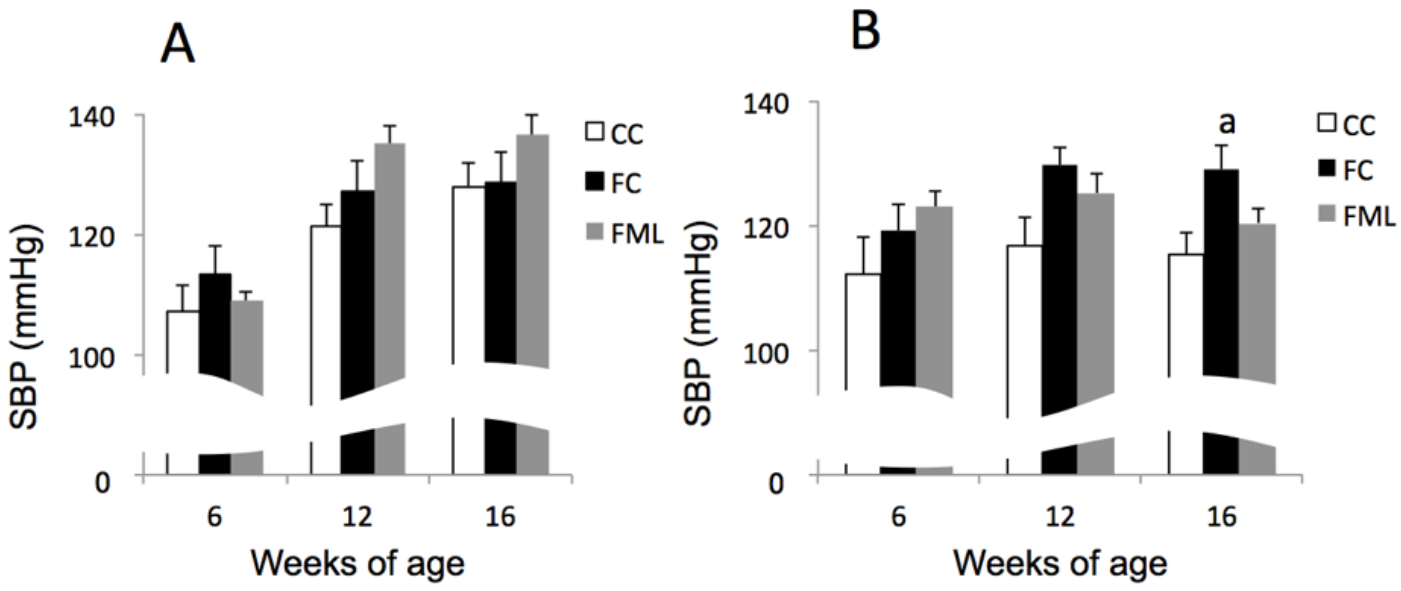

Figure 2-5. Changes in SBP of male (A) and female (B) weaned offspring. Each bar shows mean±SE (in A: CC, n=6; FC, n=5; FM, n=6; and in B: CC, n=6; FC, n=5; FM, n=5). a indicates $p < 0.05$ versus CC.

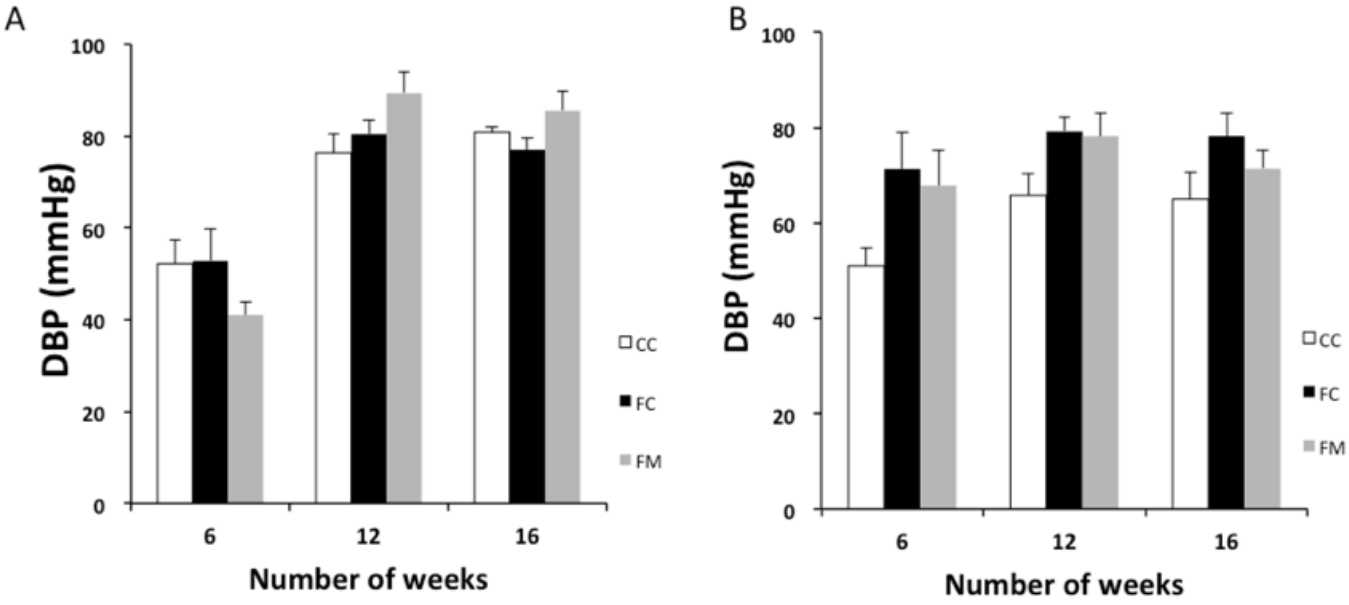

Figure 2-6. Changes in DBP of male (A) and female (B) weaned offspring. Each bar shows mean±SE (in A: CC, n=6; FC, n=5; FM, n=6; and in B: CC, n=6; FC, n=5; FM, n=5).

2.5 Discussion

Herein, we have studied the effects on the offspring of maternal fructose consumption during pregnancy and tested the hypothesis that supplementing with melinjo seed extract can offset these deleterious effects. The key findings of this study are (i) the level of renal pAMPK in FM of 17-week female offspring, but not of the male offspring, was increased after maternal MSE intake during lactation (Figures 2-2 and 2-3); (ii) maternal fructose intake down-regulated renal eNOS expression in FC group while maternal MSE consumption maintained renal eNOS expression in FM group of female offspring but not of the male offspring (Figure 2-4); and (iii) maternal MSE intake during lactation lowered the SBP in FM of 17-week female offspring, but not of the male offspring (Figure 2-5).

Previous researches have already demonstrated that the risks of a number of chronic diseases in adulthood may have originated before birth in the intrauterine environment (Harding 2001). However, improving maternal nutrition through dietary interventions can benefit the offspring in later life (Joshi et al 2003). In this study, the significant changes in the 17-week female FC offspring are consistent with the reported maternal fructose consumption effects. In addition, our results showed that maternal MSE supplementation during lactation ameliorated the fructose-induced adverse metabolic effects.

eNOS serves many functions in the vasculature. Mainly, it is responsible for the production of nitric oxide (NO), an important endogenous vasodilator. Induced by stimuli such as shear stress, AMPK activates eNOS, which leads to the production of NO from L-arginine (Tsuchiya et. al 2013; Klahr 2001). This NO is released across the endothelium into the neighbouring smooth muscles stimulating vasodilation (Klahr 2001) and reducing blood pressure (Xia et al 2014). In the kidney, a decrease

in the production and biological action of NO are linked to the elevation of arterial pressure, and conversely, an increased activity may represent a compensatory mechanism to mitigate the hypertension (Lee 2008). In this study, the phosphorylation of AMPK in the kidney for the FM group of 17-week female offspring was upregulated compared with CC and FC (Figure 2-3B). As activation of AMPK activates eNOS, the level of eNOS in the FM group was significantly higher compared with FC (Figures 2-3B and 2-4B), which was significantly lower compared with CC. Consequently, FC showed a significantly higher SBP compared with CC while FM remained to have SBP levels same as CC.

Fructose has been reported to lower the activity and expression of eNOS (Klein et al 2015). Conversely, resveratrol, a polyphenolic compound, is known to enhance eNOS expression and activity (Xia et al 2014). MSE mainly contains dimer resveratrol and few amounts of trans-resveratrol. Although the exact mechanism remains unclear, our results suggest that maternal MSE consumption during lactation may have enhanced the expression of eNOS resulting in the lowering of SBP of FM group of 17-week female offspring (Figure 2-5B).

Interestingly, these results are only evident in the female offspring. While the exact reason as to why the changes observed are gender specific is still unclear, other similar studies have also reported the same observation. Vickers et al (2011) demonstrated that maternal fructose ingestion during pregnancy significantly increased circulating plasma fructose and leptin levels with reduced placental weights of female fetuses but this was not observed in male fetuses. In another study by Mukai et al (2013), maternal fructose intake during pregnancy modulates hepatic and hypothalamic AMPK signaling in female offspring but not in male.

In the same way, our results showed that female offspring were more vulnerable to the effects of placental fructose and MSE suggesting sex-specific sensitivities. There are other studies that report sex-dependent placental growth and function. Clifton (2010) showed that female placenta was more responsive to changes in glucocorticoid concentration in both preterm and term pregnancies while male placenta appeared to be resistant. It can be said that in the present study, there is also a potential difference in the placental growth and function between male and female. This difference resulted in disparity in the transfer of nutrients and growth factors within the placenta giving different fates for the male and female offspring in later life.

2.6 Conclusion

Our data demonstrate that MSE consumption during lactation improved vasodilation and attenuated the development of hypertension in the 17-week female offspring of fructose-fed pregnant rats. This improved vasodilatory function is associated with enhanced eNOS expression in the kidney via increased AMPK phosphorylation. According to our findings, melinjo seed extract supplementation during lactation may improve vasodilation and attenuate hypertension in female offspring in later life.

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CHAPTER III.
Cytotoxic Effects of Melinjo Seed Extracts on Human Cancer Cells

3.1 Abstract

Cancer remains to be one of the leading causes of death worldwide. However, there is still no drug that is found to be completely effective and safe. Thus, the search for new anti-cancer drugs that will be more selective and have lesser side effects is still a major challenge. For this reason, most recent research works on cancer drug discovery focus on plants and plant-derived natural products like polyphenols. In the present study, *in vitro* screening of MSE against a panel of human cancer cells revealed its cytotoxicity against HepG2 liver cancer cells, HeLa cervical cancer cells, OSRC-2 renal cancer cells, and H460 lung cancer cells. The obtained IC_{50} values for HepG2, HeLa, OSRC-2, and H460 are 172, 328, 411, and 457 μg/mL, respectively. Among the four tested human cancer cell lines, HepG2 was found to be the most sensitive to MSE with the IC_{50} value of 172 μg/mL. Accordingly, the effect of MSE treatment in HepG2 cells was further investigated to determine its mechanism and action.

3.2 Introduction

Cancer remains to be the one of the leading causes of death worldwide and still, the number of cases is increasing. According to the World Health Organization (WHO), approximately 14 million new cases of cancer are diagnosed per year and, among these, more than 60% result in death (8.8 million people in 2015) (WHO, 2017). It is a major public health burden that affects both developed and developing countries.

Cancer is a general term used to describe a series of malignant diseases that may affect various parts of mammalian body (Poonam and Chandana, 2015). It is a complex disease wherein cells do not respond anymore to the signals that regulate cellular growth and death (O'Connor and Adams, 2010). It is characterized by quick and uncontrolled formation of abnormal cells, which can form a growth or tumor by accumulating together (Poonam and Chandana, 2015). In addition, it can proliferate all throughout the body, affecting other areas by initiating abnormal growth. Normal cells become cancerous due to the mutations that build up in different genes that regulate cell proliferation (O'Connor and Adams, 2010). This originates within the tissues and as they progress and divide, their growth deviates further from normalcy, making them increasingly resistant to controls and evade programmed cell death (O'Connor and Adams, 2010).

Treatment of cancer involves surgery, chemotherapy, hormonal therapy, radiation therapy, immunotherapy, and cancer chemotherapeutic agents (Sudhakar A, 2009; Poonam and Chandana, 2015). Through the years, much effort has been focused on the synthesis and discovery of potential anticancer drugs. Several drugs are now currently available in the market to cure different types of cancer but still, no drug is found to be completely effective and at the same time safe. For an anti-

cancer drug to be successful, it should eradicate cancer cells while avoiding excessive damage to normal cells. This however, is the main challenge as it is difficult to attain, because most existing anti-cancer drugs have prolonged toxicity and adverse side effects. For this reason, most recent research works on cancer drug discovery focus on plants and plant-derived natural products like polyphenols. It is estimated that approximately 50-60% cancer patients in the United States use plant-derived agents (complementary and alternative medicine), exclusively or alongside traditional therapeutic regimen such as chemotherapy and/or radiation therapy (Wang et al., 2012).

Numerous natural products including polyphenols and their derivatives are found to be potent anti-cancer agents. These include curcumin from tumeric, genistein from soybean, tea polyphenols from green tea, resveratrol from grapes, sulforaphane from broccoli, isothiocyanates from cruciferous vegetables, silymarin from milk thistle, diallyl sulfide from garlic, lycopene from tomato, rosmarinic acid from rosemary, apigenin from parsley, and gingerol from gingers (Wang et al., 2012). Melinjo seed extracts (MSE) were found to be rich in polyphenols such as dimeric stilbenoids, including gnetin C, gnemonosides A and D, and a few amounts of trans-resveratrol. A recent study has reported the anti-cancer and anti-tumor activity of MSE (Narayanan et al., 2015). Their results indicated that MSE significantly inhibited the proliferation of pancreatic, prostate, breast and colon cancer cell types (PANC-1, AsPc-1, Pan-02, PC-3, DU-145, LNCaP, PTEN-CaP8, MCF-7, HT-29 and Colon-26) without affecting normal cells (HEK-293T and RWPE-1). This anti-tumor activity was validated *in vivo,* showing that 50 and 100 mg/kg per day of MSE administration significantly inhibited tumor growth, intratumoral angiogenesis, and liver metastases in BALB/c mice bearing colon-26 tumors (Narayanan et al, 2015).

MSE has already been reported to have anti-cancer properties. However, its reported cytotoxicity is still limited to pancreatic, prostate, breast and colon cancer cell types. In the present study, the cytotoxicity of MSE against liver (HepG2), cervical (HeLa), lung (H460) and kidney (OSCR2) cancer cells was determined via MTT Assay. This was done to further investigate the anti-cancer efficacy of MSE, which can be used for future studies in the prevention, or treatment of certain cancer.

3.3. Materials and Methods

3.3.1. Materials

The MSE powder (Lot. No. YMP-M-130610) containing 0.05% trans-resveratrol, 1.25% gnetin C, 20.84% gnemonoside A, 3.82% gnemonoside D and 9.0% dextrin was obtained from Yamada Bee Company, Inc. (Okayama, Japan). It was dissolved in water at up to 50 mg/mL (stock solution). HepG2 liver cancer cells (Health Protection Agency Culture Collections, Philadelphia, USA); H460 lung cancer cells (ATCC HTB-177, Manassas, Virginia); HeLa cervical cancer cells (Riken Cell Bank, Tsukuba, Japan); and OSRC-2 kidney cancer cells (Riken Cell Bank, Tsukuba, Japan) were used for the study. Cells were maintained in RPMI-1640 medium (Wako) with 10% fetal bovine serum (FBS) (Biosera; Kansas City, MO, USA).

3.3.2. Cell culture

Cells were cultured in RPMI-1640 medium (Wako) with 10% FBS in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were pre-incubated in 25-cm^2 flasks (Thermo Fisher Scientific; Waltham, MA, USA).

3.3.3. MTT Assay

A standard MTT assay was performed based on the method reported by Mosmann (1983). Cells were seeded into 96-well plates at a density of 1.0×10^4 cells per well and maintained at 37 °C with 5% CO_2. After 24 h, the cells were treated with various concentrations of MSE (0, 0.005, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5 1.0, 2.5 and 5.0 mg/mL) and incubated for 72 h under the same conditions. After incubation, the medium was aspirated and replaced with 100 µL of MTT in RPMI- 1640 medium with 10% FBS. The cells were then incubated at 37 °C with 5% CO_2. After 3 h, the MTT solution was aspirated followed by the addition of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. The plate was then incubated for 10 min followed by measurement of the absorbance at 570 nm using a Thermo Labsystems Multiskan JX plate reader. Cisplatin (SigmaAldrich; Darmstadt, Germany) was used as the positive control.

3.4 Results

3.4.1. MSE inhibits the proliferation of human cancer cells

The anticancer potential of MSE was determined against a panel of human cancer cell types (HepG2, H460, HeLa, and OSRC-2) using MTT assay. As shown in Figures 3-1 to 3-4, MSE inhibited the proliferation of these cancer cells in a dose-dependent manner. The IC_{50} values are summarized in Table 3-1. Among all the tested cells, HepG2 showed the highest sensitivity to MSE with the IC_{50} value of 171.5 µg/mL.

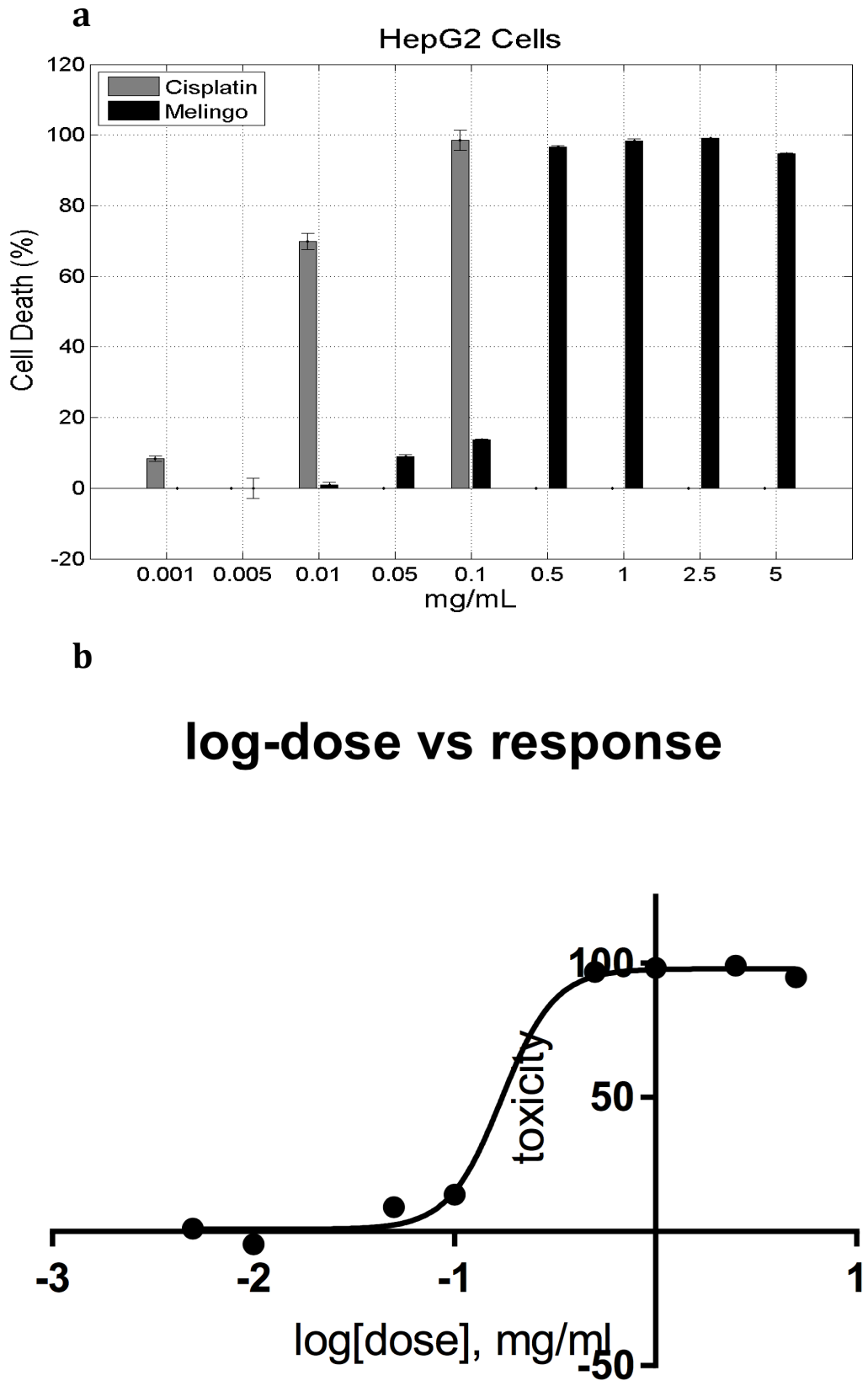

Figure 3-1. Effect of MSE on HepG2 cell proliferation. (a) Cell Death (%) (b) log dose vs response curve

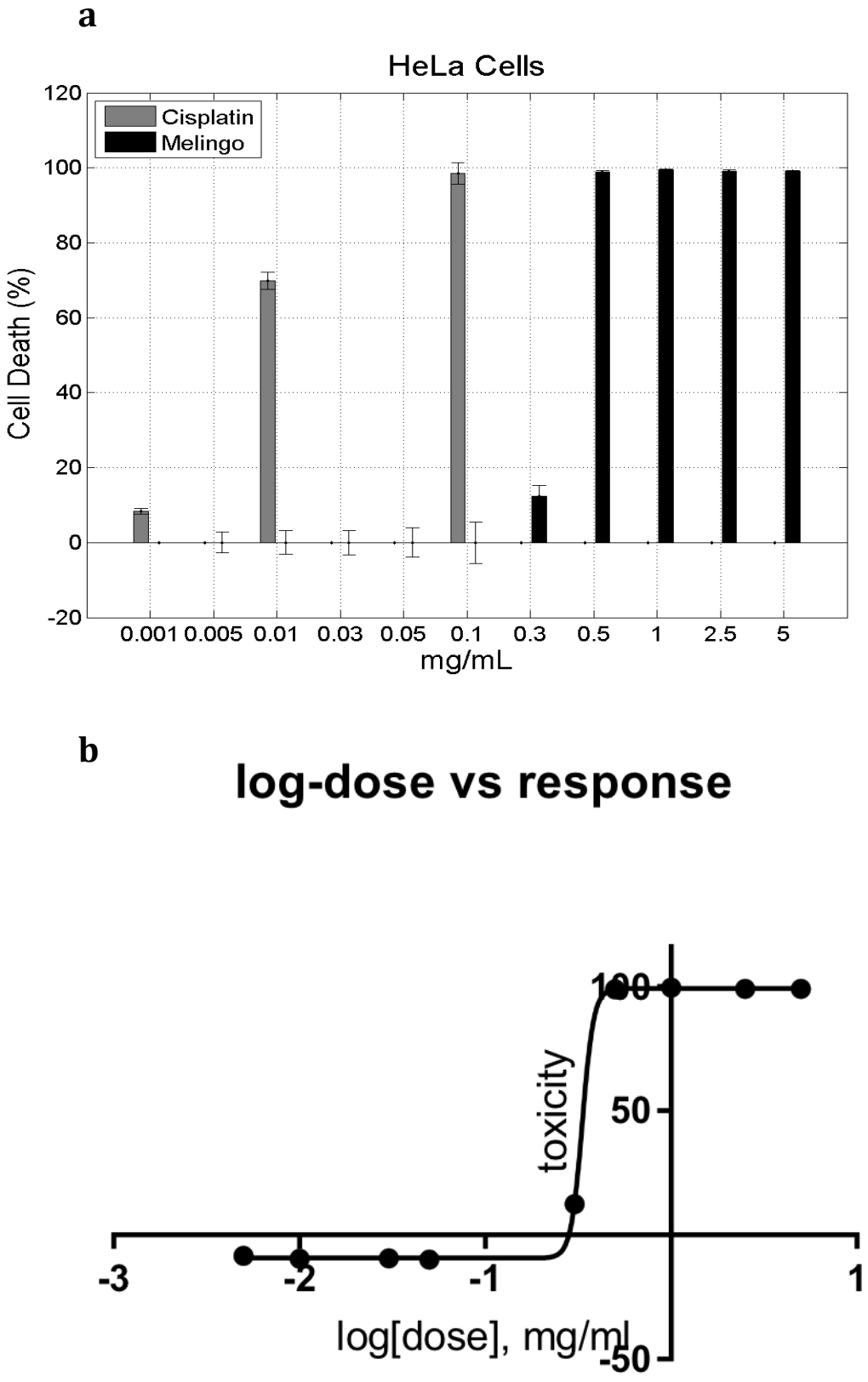

Figure 3-2. Effect of MSE on HeLa cell proliferation. (a) Cell Death (%) (b) log dose vs response curve

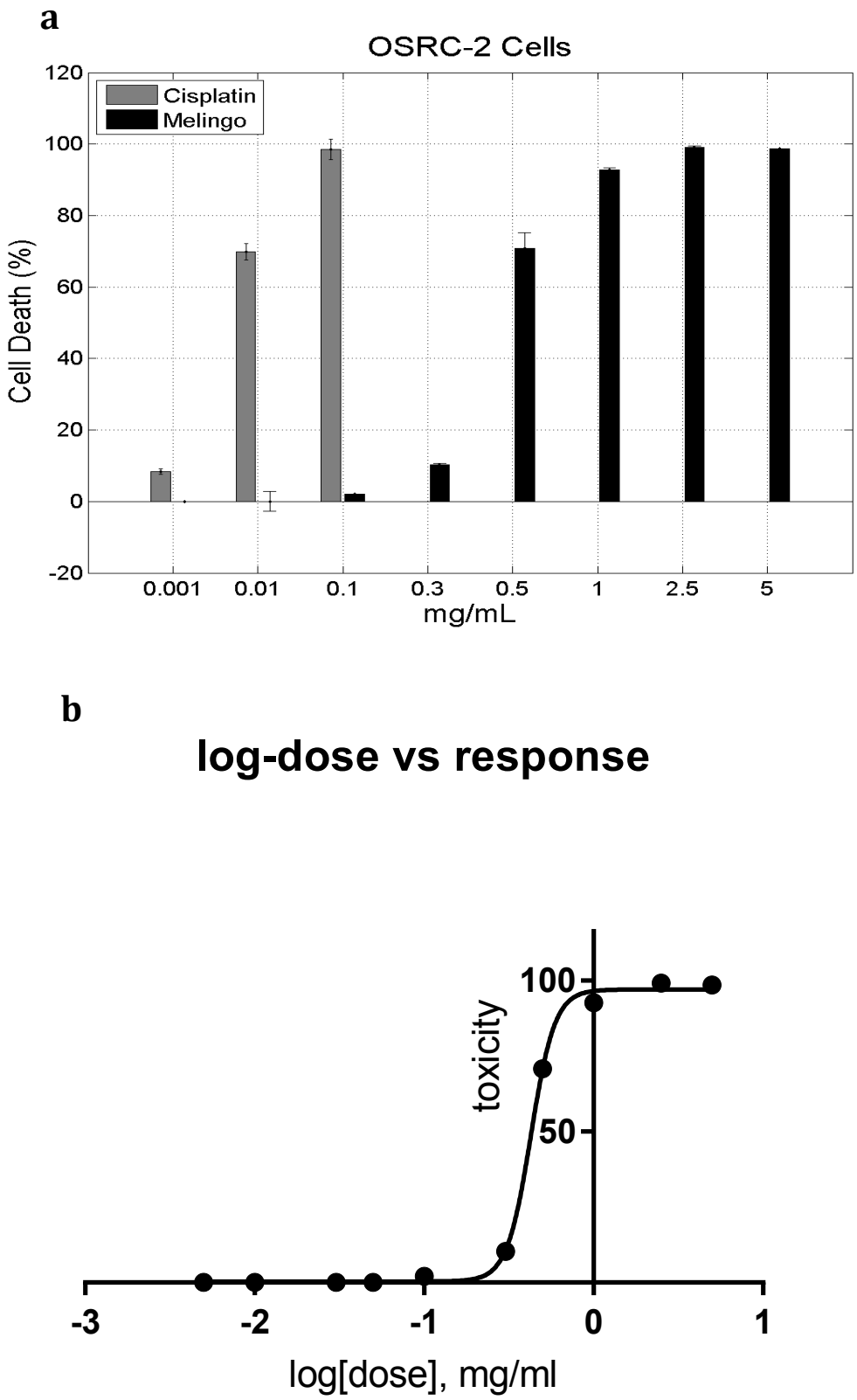

Figure 3-3. Effect of MSE on OSRC-2 cell proliferation. (a) Cell Death (%) (b) log dose vs response curve

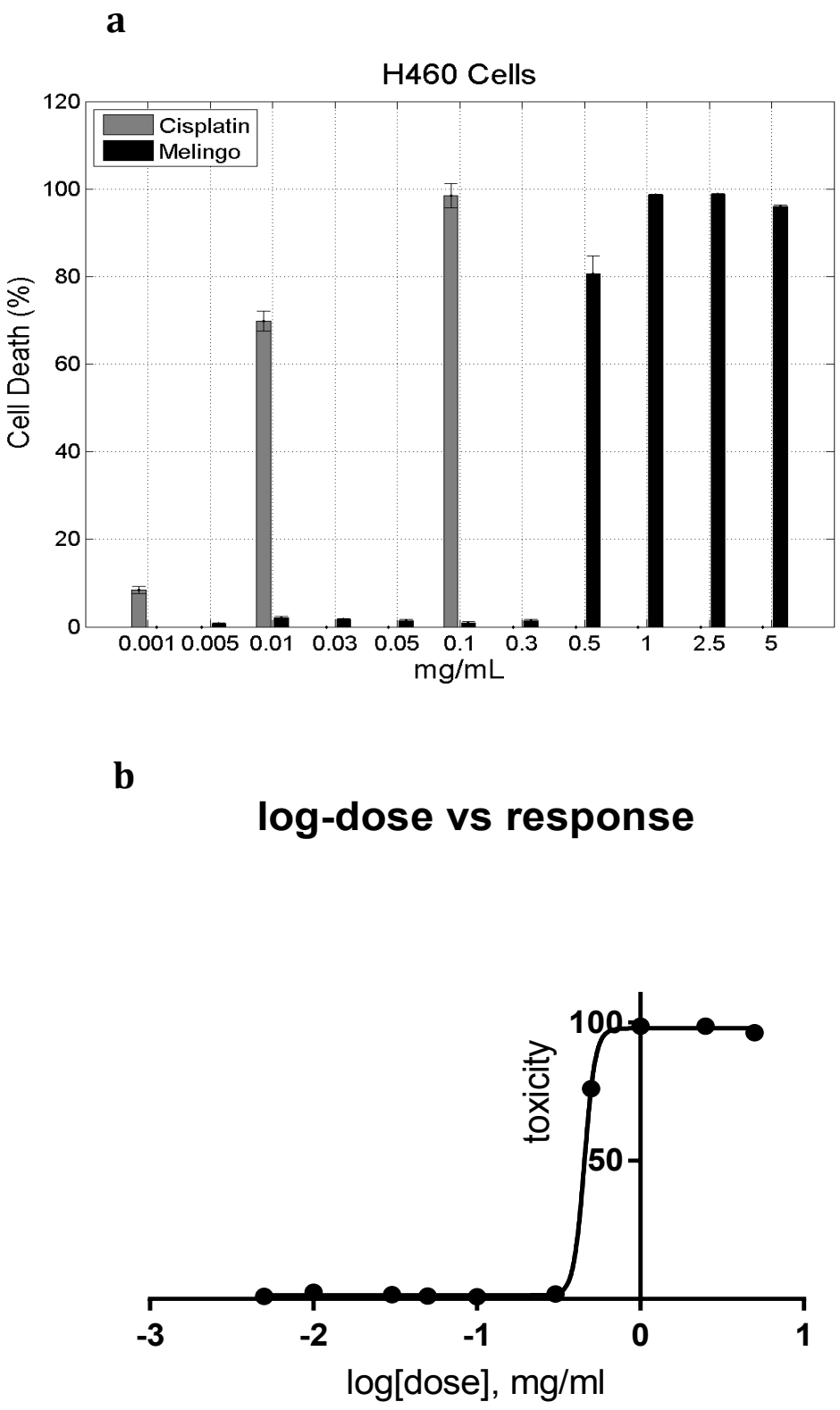

Figure 3-4. Effect of MSE on H460 cell proliferation. (a) Cell Death (%) (b) log dose vs response curve

Table 3-1. IC_{50} values of MSE against cancer cell proliferation

Cell lines	Description	IC_{50} (μg/mL)
HepG2	Human hepatic cancer cells	170
HeLa	Human cervical cancer cells	330
OSRC-2	Human renal cancer cells	410
H460	Human lung cancer cells	460

3.5 Discussion and Conclusion

In this chapter, the anti-cancer potential of MSE in a panel of human cancer cells has been confirmed. The obtained IC_{50} values for HepG2, HeLa, OSRC-2, and H460 are 171.5, 327.8, 410.5, and 457.2 μg/mL, respectively. Since plant extracts have been demonstrated to be an effective natural source of anti-cancer therapy, in this chapter we proved the anti-proliferation effect of MSE using MTT assay. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is a widely used protocol for assessment of cytotoxicity, cell viability, and proliferation studies in cell biology (Stockert et al., 2012). It is based on the conversion of yellowish MTT into an insoluble violet-blue formazan by dehydrogenase and reducing agents present in metabolically active cells, which determines mitochondrial activity (Stockert et al., 2012; Bandopadhyaya et al., 2015). Since generally, the total mitochondrial activity is related to the number of viable cells, MTT assay is utilized to measure the *in vitro* cytotoxic effects of potent drugs on cell lines.

Table 3-2 provides the IC_{50} values of MSE against different cancer cells as reported by Narayanan et al. From the table, you can see that the reported values are much lower than the values obtained from this study. However, it should be noted that the protocol used by the authors were different from the MTT assay used in this study. The authors used MTS assay, while the theory is still the same, notably, they

utilized 3 x 10^3 cells per well in their assay. Whereas, in the present study, we used 1.0 x 10^4 cells per well. This big difference in the cell count per well is the reason for the difference in the magnitude of IC_{50} values obtained in the two studies. Nevertheless, the main objective of this chapter is to screen MSE against various cancer cells and choose among them the cell line that is most sensitive to MSE, which will be used for further analysis for the determination of its mechanism of action. From the obtained results, hepatocarcinoma cells (HepG2) are the most sensitive to MSE. As such, HepG2 was chosen for further analysis to determine the type of cell death it underwent and its detailed mechanism.

Table 3-2. IC_{50} values of MSE against cancer cell proliferation (Narayanan et al., 2015)

Cell lines	Description	IC_{50} (µg/mL)
PANC-1	Human pancreatic cancer cells	61.27
AsPC-1	Human pancreatic cancer cells	53.74
PC-3	Human prostate cancer cells	38.26
DU-145	Human prostate cancer cells	39.38
LNCaP	Human prostate cancer cells	34.26
MCF-7	Human breast cancer cells	37.3
HT-29	Human colon cancer cells	39.33

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CHAPTER IV.

Melinjo (*Gnetum gnemon* L.) Seed Extracts Induced Apoptosis in HepG2 Cells via Inhibition of NF-κB Pathway

4.1. Abstract

Melinjo (*Gnetum gnemon* L.) seed extracts (MSE) were found to be rich in polyphenols such as dimeric stilbenoids, including resveratrol dimers: gnetin C, gnemonosides A and D and a few amounts of *trans*-resveratrol. Previous studies reported its various pharmacological activities including anti-tumor activity against various cancer cells *in vitro*. However, the exact molecular mechanism involved in MSE-induced cancer cell toxicity remains largely unknown, particularly in human hepatocellular carcinoma (HepG2) cells. In the present study, the effect of MSE treatment in HepG2 cells was investigated. In MTT assay, we found that MSE dose- and time-dependently induced cytotoxicity in HepG2 cells. Annexin V-FITC/PI staining analysis by flow cytometry and DNA ladder analysis indicated that MSE induced apoptosis in a dose-dependent manner. This apoptotic effect of MSE was found to be associated with reactive oxygen species generation suggested by the dose-dependent depletion of intracellular free-SH levels. Western blot data showed the modulation of Bcl-2 family of proteins, cytochrome c release and cleavage of caspase 3 implying the induction of mitochondrial apoptotic pathway. In addition, MSE inhibited the activation of the prosurvival NF-κB pathway via induction of dephosphorylation and up-regulation of IkB-α, which ultimately leads to the inhibition of the translocation of NF-κB p65 to the nucleus. In conclusion, our results provide evidence for the first time that MSE has potent anticancer activity against HepG2 cells, thus, providing basis for future clinical application of MSE in liver cancer cases.

4.2 Introduction

Incidence rates of hepatocellular carcinoma are on the rise due to increasing occurrence of hepatitis B (HBV), hepatitis C (HCV), cirrhosis and non-alcoholic fatty liver disease (Flores et al, 2014; Raphael et al, 2012). According to the World Health Organization (WHO), liver cancer is one of the most common causes of cancer death in the world leading to 788,000 deaths in 2015 (WHO, 2017). The choices of treatment for liver cancer include curative treatments such as surgical resection, liver transplantation, and percutaneous ablation; and palliative approaches such as immunotherapy, hormonal compounds and systematic chemotherapy (Raphael et al, 2012). Despite recent improvements in surgical techniques and chemotherapy, a significant amount of research is still needed to develop other agents that may help improve outcomes. Specifically, there is a need to develop effective chemotherapeutic drugs that selectively targets cancer cells without adverse side effects on normal cells.

Plant-derived polyphenols exhibited anticarcinogenic properties, which include inhibitory effects on cancer cell proliferation, tumor growth, angiogenesis, metastasis, inflammation, and apoptosis induction (Niedzwiecki et al, 2016). Melinjo (*Gnetum gnemon* L.) is a plant that is widely cultivated in Southeast Asia. Melinjo seed extracts were found to be rich in polyphenols such as dimeric stilbenoids, including gnetin C (resveratrol dimer), gnemonosides A and D, and a few amounts of trans-resveratrol as shown in (Kato et al, 2009). Melinjo seeds have been reported to be safe for use as ingredients in functional foods or pharmaceuticals (Tatefuji et al, 2014).

Recent studies reported various pharmacological activities of MSE. Serum uric acid levels of non-obese Japanese males were decreased by MSE after 4 and 8 weeks

of administration (Konno et al, 2013). Ethanol extracts of melinjo seeds showed radical scavenging effect, lipase and α-amylase inhibition activity, and antimicrobial activity against food microorganisms and enterobacteria (Kato et al, 2009). MSE intake during lactation stimulated hepatic AMP-activated protein kinase (AMPK) in the offspring of excessive fructose-fed pregnant rats (Kataoka et al, 2016). MSE treatment of high fat diet-fed mice improved survival and reduced the risk of death by 25%, indicating improvement of several aspects of metabolic syndrome (Ikuta et al, 2015). MSE also demonstrated angiotensin converting enzyme inhibitory activity (Mun'im et al, 2017). Gnetin C, a resveratrol dimer isolated from MSE showed inhibitory effects on tyrosinase activity and melanin biosynthesis (Yanagihara et al, 2012).

In addition, MSE suppressed multiple angiogenesis-related endothelial cell functions and/or tumor angiogenesis indicating its potential anticancer property (Kunimasa et al, 2011). MSE also showed antitumor activity against various human and murine tumor models in vitro (Narayanan et al, 2015). These results showed that MSE significantly inhibited the proliferation of pancreatic, prostate, breast and colon cancer cell types without affecting normal cells. This anti-tumor activity was validated in vivo, showing that 50 and 100 mg/kg per day of MSE administration significantly inhibited tumor growth, intratumoral angiogenesis, and liver metastases in BALB/c mice bearing colon-26 tumors (Narayanan et al, 2015).

Although MSE has been studied against various cancer cells, there is no study reporting its effect on human hepatocellular carcinoma cells. Moreover, there is still no comprehensive investigation on the exact mechanism on how MSE induce apoptosis on any cancer cell line. In the present study, we investigated the anticancer potential of MSE against liver cancer cells (HepG2). In vitro study showed

a dose-dependent cytotoxicity of MSE against HepG2 cells, inducing mitochondrial apoptosis via oxidative stress resulting in the inhibition of the activity of the pro-survival factor, nuclear factor kappa B (NF-κB). These results suggest that MSE is a potent material for liver cancer therapy and can provide basis for clinical application of MSE in liver cancer cases.

4.3. Materials and Methods

4.3.1. Chemical agents

The MSE powder (Lot. No. YMP-M-130610) containing 0.05% *trans*-resveratrol, 1.25% gnetin C, 20.84% gnemonoside A, 3.82% gnemonoside D and 9.0% dextrin was obtained from Yamada Bee Company, Inc. (Okayama, Japan). It was dissolved in water at up to 50 mg/ml (stock solution). HepG2 cells were purchased from Health Protection Agency Culture Collections (Philadelphia, USA). Cells were maintained in RPMI-1640 medium (Wako) with 10% fetal bovine serum (FBS) (Biosera; Kansas City, MO, USA). Trypan Blue Dye 0.4% solution and western blotting marker were purchased from BioRad (Hercules, CA, USA). LDH Activity was measured using Cytotox 96® Non-Radio Cytotoxicity Assay from Promega (Fitchburg, WI, USA). DNA was isolated using High Pure PCR Template Preparation kit from Roche Diagnostics (Mannheim, Germany). Cytochrome c was quantified using cytochrome c release apoptosis assay kit (Merck-Millipore; Darmstadt, Germany). The following antibodies were used for western blotting: NF-κB (ab16502, Abcam), p-IKB (2859S, Cell Signaling), IKB (48145, Cell Signaling), Bax (SC-7480, Santa Cruz Biotechnology, Inc.), Cytochrome C (612503, Biolegend), BCl-2 (PC68, Oncogene) and Cleaved caspase 3 (9661, Cell Signalling). Anti-rabbit IgG, HRP-Conjugated (W40113,

Promega), anti-mouse IgG HRP-linked antibody (7076, Cell Signaling), StrepTactin HRP-Conjugate (BioRad) and Amersham™ ECL™ western blotting detection reagents (GE Healthcare; Buckinghamshire, UK) were all used for western blotting detecton. All other chemicals used were of analytical grade.

4.3.2. Cell culture

HepG2 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific; Waltham, MA, USA) with 10% FBS in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were pre-incubated in 25-cm^2 flasks (Thermo Fisher Scientific). After 24 h, the medium was removed and replaced with or without various concentrations of MSE, and incubated for 48 h.

4.3.3. MTT assay

A standard MTT assay was performed based on the method reported by Mosmann (1983). HepG2 cells were seeded into 96-well plates at a density of 1.0 × 10^4 cells per well and maintained at 37 °C with 5% CO_2. After 24 h, the cells were treated with various concentrations of MSE (0, 0.1, 0.3, 0.4, 0.5, 1.0 and 5.0 mg/mL) and incubated for 24, 48 and 72 h under the same conditions. After incubation, the medium was aspirated and replaced with 100 µL of MTT in RPMI- 1640 medium with 10% FBS (0.5 mg/mL). The cells were then incubated at 37 °C with 5% CO_2. After 3 h, the MTT solution was aspirated followed by the addition of DMSO to dissolve the formazan crystals. The plate was then incubated for 10 min followed by measurement of the optical density at 570 nm using a Thermo Labsystems Multiskan

JX plate reader. Cisplatin (SigmaAldrich; Darmstadt, Germany) was used as the positive control.

4.3.4. Cell viability

Trypan blue exclusion assay was performed to measure the cell viability. HepG2 cells were incubated in RPMI-1640 medium with 0, 0.1, 0.3, 0.5 and 1.0 mg/mL MSE for 48 h. After incubation, cells were stained with 0.25% trypan blue solution in phosphate-buffered saline. Total cells and trypan blue stained cells were measured using a haemacytometer (BioRad; Hercules, CA, USA). Cell viability was expressed as a percentage against the total cell number in each experiment. To ensure reproducibility, each experiment was done at least three times.

4.3.5. Lactate dehydrogenase (LDH) activity assay

Using Cytotox 96® Non-Radio Cytotoxicity Assay kit, the cytotoxicity was evaluated by measuring the LDH activity in the treatment medium as described by Rahman et al (2017). HepG2 cells were cultured in the medium with or without MSE (0.1, 0.3, 0.5, and 1.0 mg/mL) for 48 h. After incubation, 50 µL of the medium was transferred to a 96-well plate followed by the addition of a 50 µL tetrazolium-containing substrate mixture. The mixture was allowed to react for 30 min at room temperature (25°C). After incubation, stop solution (50 µL) was added, and the formazan dye formation was quantified by absorbance measurement at 490 nm using a Micro Plate Reader (BioRad, model 450). LDH Activity was expressed as LDH Activity/1 x 106 cells. The experiment was performed at least three times.

4.3.6. Apoptosis by flow cytometry

The apoptotic effect of MSE to HepG2 cells was detected by Annexin V-FITC Apoptosis Kit (BioVision Incorporated; Milpitas, CA, USA), according to the manufacturer's protocol. HepG2 cells treated with increasing concentrations of MSE were collected, washed with PBS and then re-suspended with the binding buffer. The cells were then incubated with Annexin V-FITC and Propidium Iodide (PI) for 5 min. Finally, both early (annexin V+/PI-) and late (annexin V+/PI+) apoptotic cells were measured by a fluorescence-activated cell sorting (FACS) machine (FACSCanto BD Biosciences; Franklin Lakes, NJ, USA).

4.3.7. Genomic DNA isolation and electrophoresis

After the treatment of HepG2 cells with various concentrations of MSE (0, 0.1, 0.3, 0.5 and 1.0 mg/mL), the genomic DNA was isolated using high pure PCR template preparation kit according to the manufacture's instruction. After 48 h of incubation, the cells were harvested using a trypsin/EDTA solution. Harvested cells were centrifuged at 1,500 rpm for 3 min and the precipitate was washed with phosphate-buffered saline (PBS). RNase (final concentration; 500 μg/mL) was added to the obtained DNA solution followed by incubation for 15 min at 37°C. After incubation, 500 μL of 100% ethanol and 20 μL of 3M NaOAc buffer were added and then stored overnight at -20°C freezer to precipitate the DNA. Next, the solution was centrifuged at 15,000 rpm for 7 min. The precipitated DNA was then washed with 70% ethanol and then centrifuged again for 3 min at 15,000 rpm. The isolated DNA was dried and the DNA concentration was measured.

Agarose gel electrophoresis was performed to analyze the DNA ladder formation. Around 3-5 μg of the isolated DNA with the loading dye was separated using a 1.5% agarose gel. The electrophoresis was carried out for 30 min at 100 V using a submarine-type electrophoresis system (Mupid-ex, Advance, Tokyo, Japan). After electrophoresis, DNA fragmentation was visualized by staining the gel with ethidium bromide solution under UV illumination using a Chem-iDoc XRS (BioRad). The experiment was performed in triplicate.

4.3.8. Measurement of intracellular free sulfhydryl (SH) levels

Intracellular free SH levels were measured to determine if oxidative stress is involved in the induction of apoptosis of MSE. HepG2 cells treated with MSE (0, 0.1, 0.3 and 0.5 mg/mL) were incubated for 48 h. After which, cells were harvested and washed with PBS followed by the addition of lysis buffer. The solution was sonicated in a two freeze-thaw cycle and then centrifuged at 1,500 rpm for 10 min. Using a protein assay dye reagent, the protein concentration was measured spectrophotemetrically. To determine the intracellular free SH levels, 2.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, pH 7) was added to the cell lysate and then the absorbance at 405 nm was measured using a Micro Plate Reader (BioRad, model 450).

4.3.9. Cell lysis and western blot analysis

HepG2 cells treated with MSE (0, 0.1, 0.3 and 0.5 mg/mL) were harvested and resuspended in 150 μL lysis buffer (2 mM HEPES, 100 mM NaCl, 10 mM EGTA, 0.1 μM PMSF, 1 mM Na_3VO_4, 0.1 mM Na_2MgO_4, 5 mM 2-glycerophosphoric acid, 10 μM

$MgCl_2$, 2 mM DTT, 50 mM NaF, and 1% triton X-100) for 10 min on ice. After which, the cells were sonicated for 2 cycles (30 s each) using a Branson Sonicator 250. The sonicated solution was then centrifuged at 1500 rpm for 10 min. The supernatant was collected and protein concentration was measured spectrophotometrically using protein assay dye reagent (BioRad). The protein concentration was then adjusted to equal amounts among all samples. Nuclear extracts were prepared as described by Schreiber et al (1989). Proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5%) and then transferred to nitrocellulose membrane through semidry blotting system (AE6678, ATTO, Tokyo, Japan). The membranes were blocked with 5% skimmed milk at room temperature and then incubated with primary antibody overnight at 4°C. The next day, membranes were washed three times followed by secondary incubation at 25°C for 1 h. After washing the membrane for 3 times, the protein bands were visualized using enhanced chemiluminescence. The detected bands were analyzed using a ChemiDoc XRS (BioRad). Each experiment was performed in triplicate.

4.3.10. Statistical Analysis

All data were presented as mean ± standard error of mean (SEM). Differences between two groups were tested using Student's t test; two-way ANOVA analysis was performed where indicated. Statistical significance was determined at the level of $P<0.05$.

4.4 Results

4.4.1. MSE inhibits proliferation of HepG2 cells

In this study, the potential cytotoxic effects of MSE were investigated on HepG2 using MTT and trypan blue staining assays. HepG2 cells were treated with 0-5.0 mg/mL MSE for 24-72 h and then cell viability was determined by MTT assay. The treatment of HepG2 cells with MSE resulted in a dose- and time-dependent inhibition of cell proliferation (Fig. 4-1). After 24 h incubation, the cell viability significantly lowered from 0.5 mg/mL. However, 48 h incubation significantly reduced the cell viability into half at only 0.3 mg/mL concentration. Incubation at 72 h further reduced the cell viability, indicating inhibitory effect of MSE in dose- and time-dependent manner. From these results, 48 h incubation with 0-1.0 mg/mL MSE concentration was chosen to be used for the rest of the study.

In addition, the cell viability was also determined by trypan blue exclusion assay. HepG2 cells were treated with 0-1.0 mg/mL MSE for 48 h. The number of trypan blue positive cells (red colored cells) increased as the dosage of MSE increases (Fig. 4-2A). These results translate to a decrease in the cell viability as the dose of MSE increases (Fig. 4-2B). Taking these results together, these suggest that MSE dose- and time- dependently inhibits survival and proliferation of HepG2 cells.

4.4.2. MSE increases LDH activity in HepG2 cells

The cytotoxic effects of MSE treatment on HepG2 cells were evaluated using LDH activity assay. HepG2 cells were treated with 0-1.0 mg/mL MSE for 48 h. After the incubation, the culture medium was collected followed by the measurement of LDH activity. The results showed that significant increase in the LDH activity was

observed from 0.3 mg/mL concentration of MSE (Fig. 4-3). As the MSE concentration increased to 1.0 mg/mL, significant increase in the LDH activity was also increased. These results agree with the observed results in MTT and trypan blue exlusion assays.

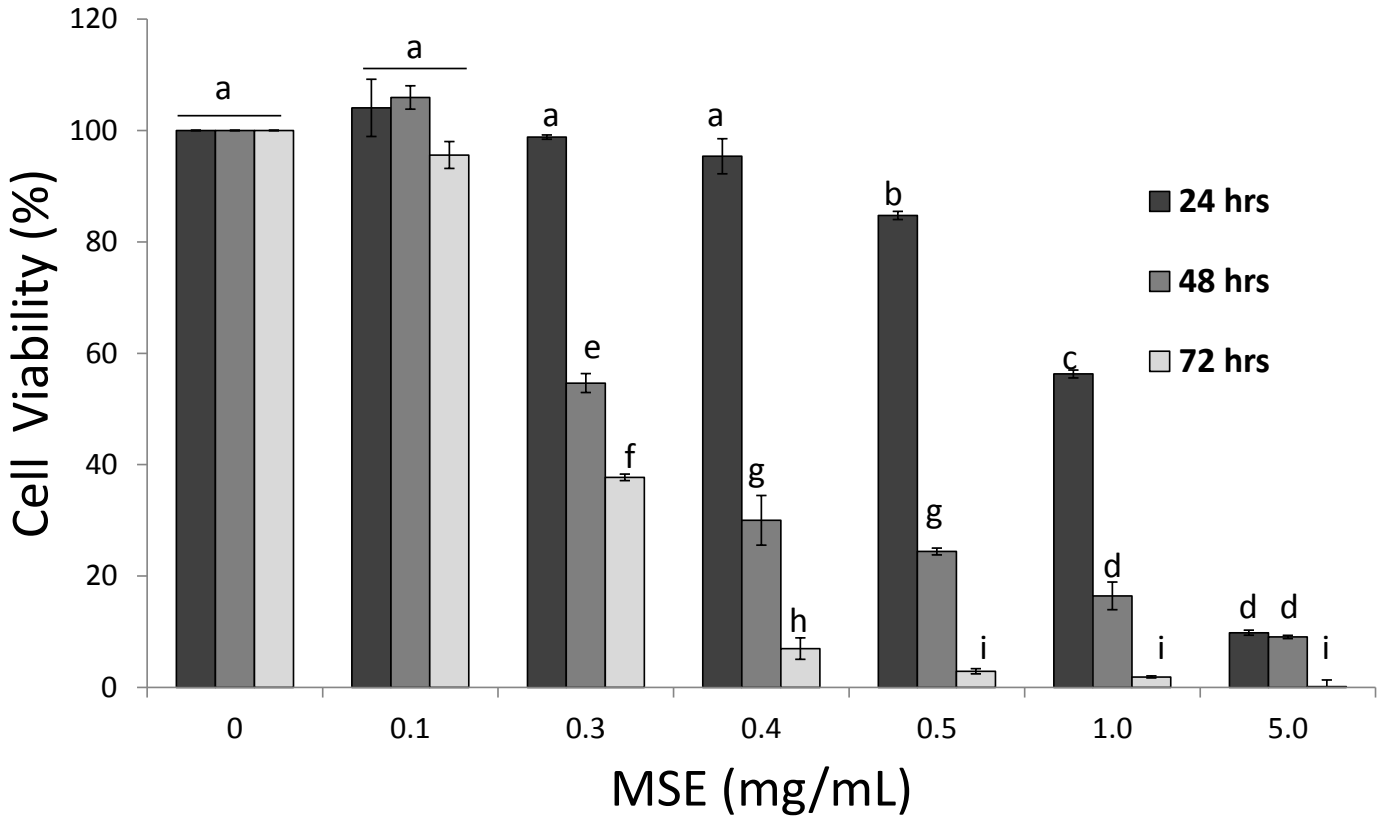


Figure 4-1. Evaluation of the effects of MSE on the proliferation and viability of HepG2 cells. HepG2 cells were seeded and cultured in 96 well plates for 24 h and treated with different concentrations of MSE for 24 h, 48 h, and 72 h. Cell viability was evaluated via MTT assay. Data are expressed as mean ± SEM of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P<0.05$).

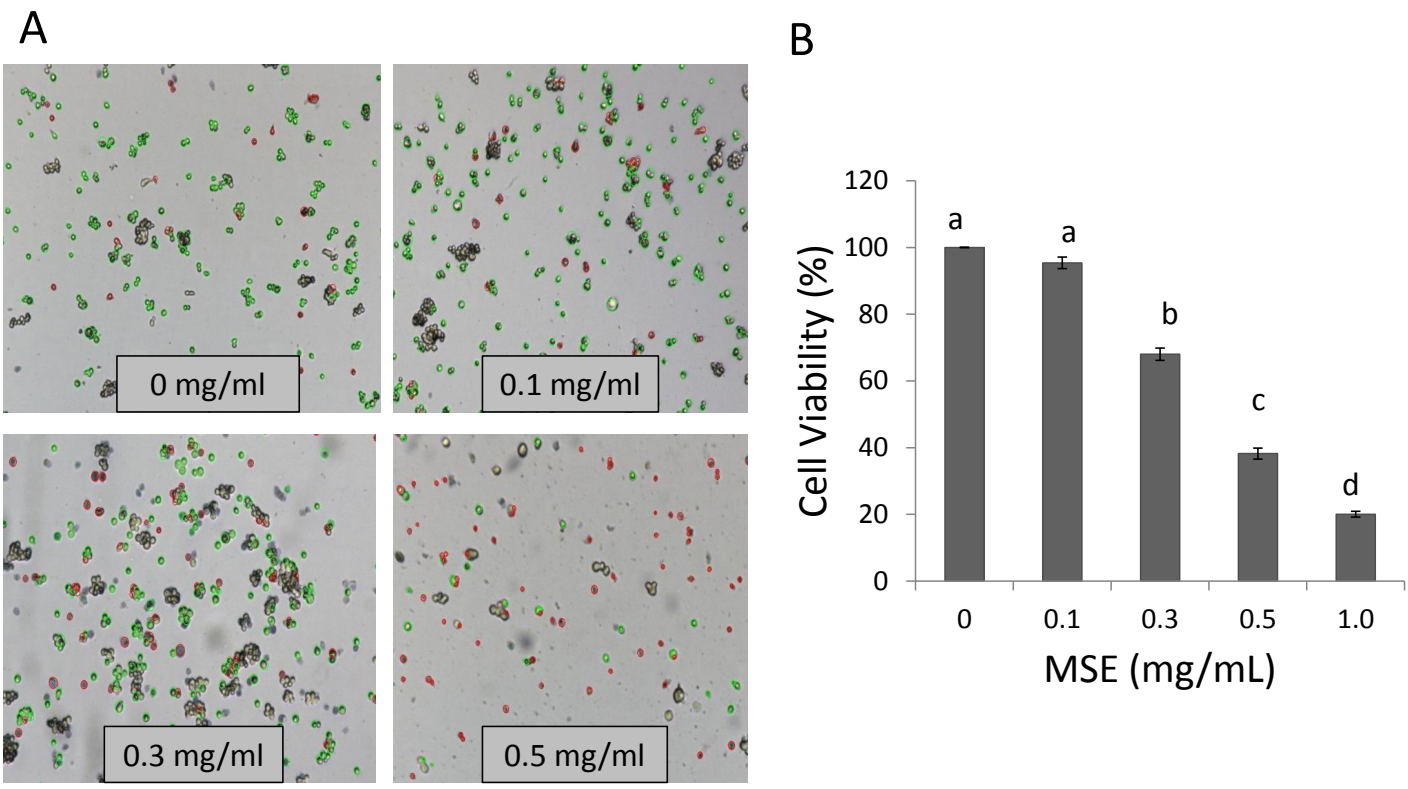


Figure 4-2. Evaluation of the effects of MSE on the viability of HepG2 cells. HepG2 cells were treated with different concentrations of MSE for 48 h. Cell viability was evaluated using trypan blue exclusion assay. (A) Representative pictures of HepG2 cells treated with different concentrations of MSE. Green colored cells are viable cells while red ones indicate dead cells. (B) Data are expressed as mean ± SEM of three or more independent experiments. Columns not sharing the same superscript letters differ significantly ($P<0.05$).

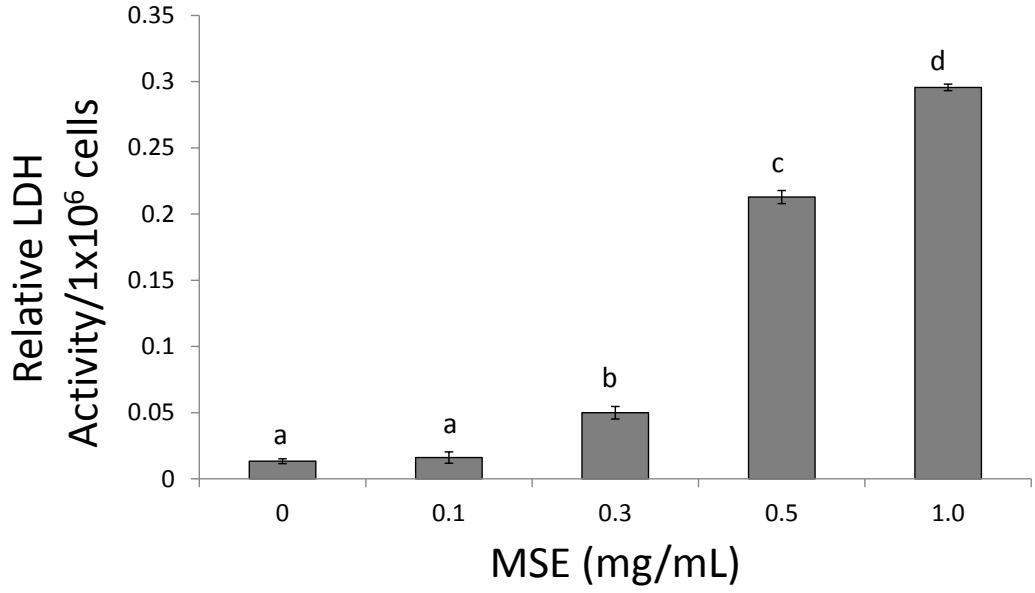
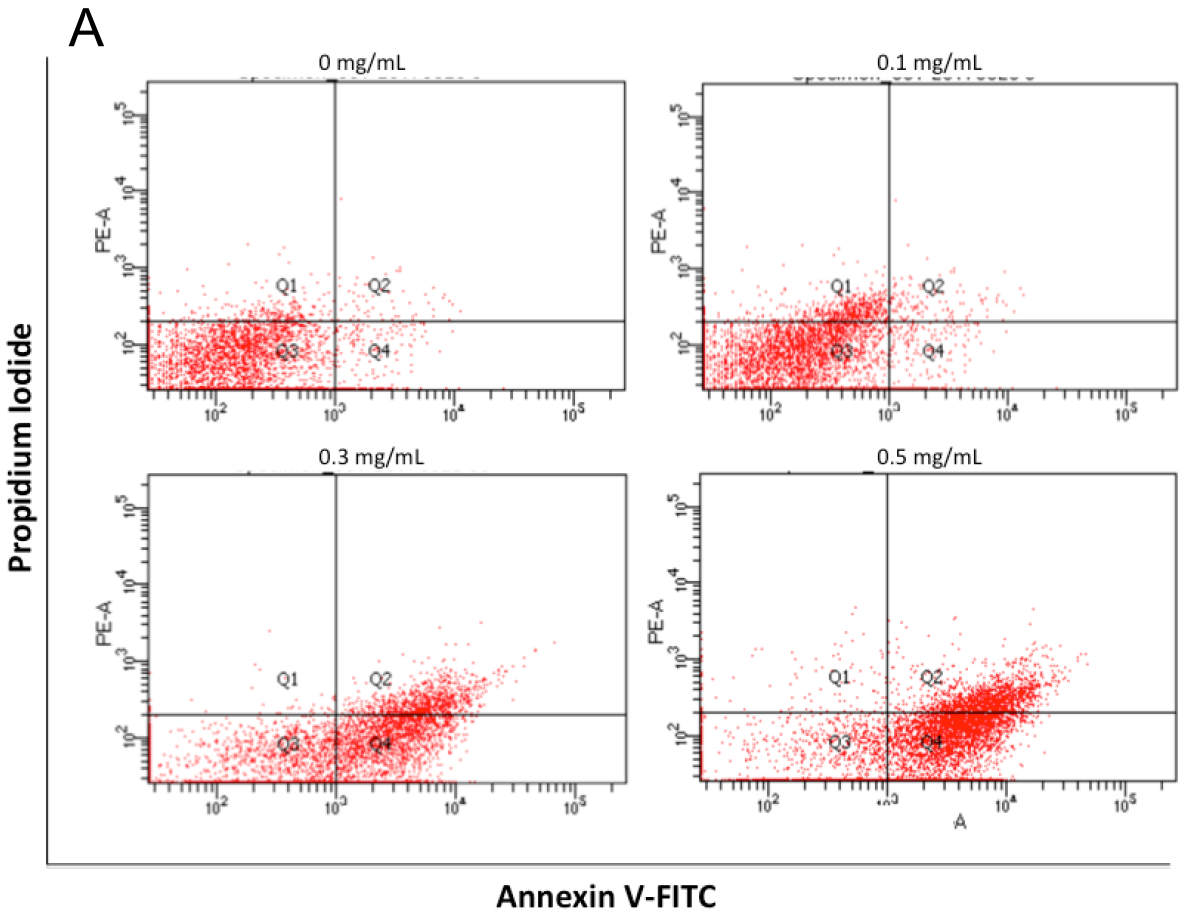


Figure 4-3. Measurement of the extracellular LDH activity in HepG2 cells. The extracellular LDH activity is used as a marker for cell membrane integrity after MSE treatment for 48 h. Data are expressed as mean ± SEM of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P<0.05$).



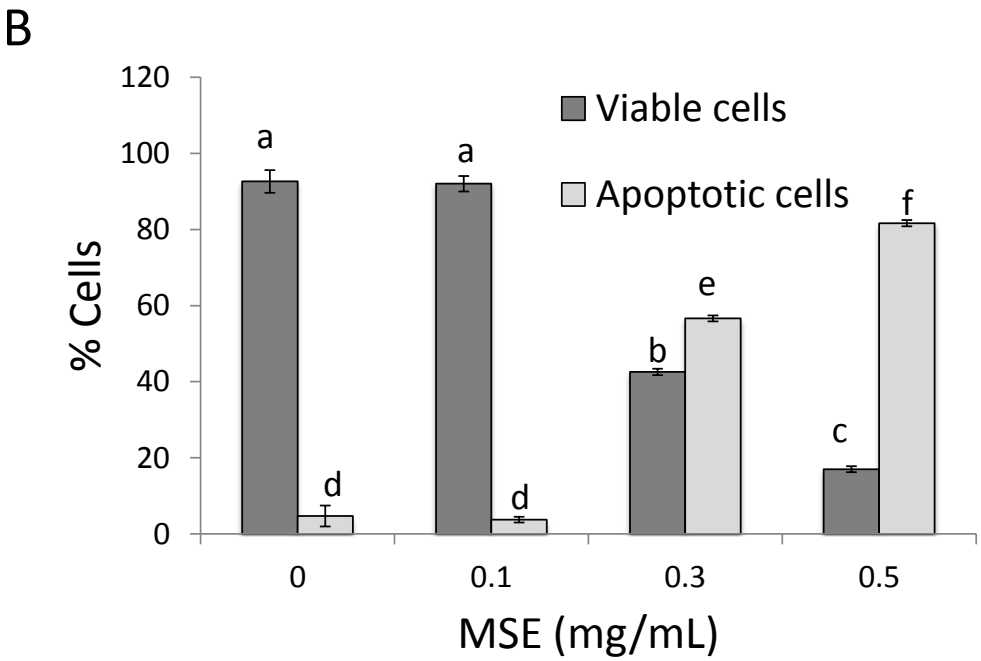


Figure 4-4. Flow cytometry analysis of HepG2 cells. (A) Cells treated with different concentrations of MSE were stained with Annexin V-FITC and PI followed by analysis of apoptosis through flow cytometry. (B) Data are expressed as mean ± SEM of three independent experiments. Columns not sharing the same superscript letters differ significantly ($P<0.05$).

4.4.3. MSE induces apoptosis in HepG2 cells

The apoptotic effect of MSE on HepG2 cells was confirmed by flow cytometry analysis. Cells treated with increasing concentrations of MSE were stained with Annexin V-FITC and PI for the determination of apoptosis. As shown in Fig. 4-4, MSE induced apoptosis in HepG2 cells in a dose-dependent manner. Results indicated that MSE induced both early (annexin V+/PI-) and late (annexin V+/PI+) apoptosis in HepG2 cells (Fig. 4-4A).

4.4.4. MSE induces DNA fragmentation in HepG2 cells

Cleavage of chromosomal DNA into oligonucleosomal size fragments is a biochemical hallmark of apoptotic cell death (Hua and Xu, 2000). These fragments can be visualized as DNA ladders via agarose gel electrophoresis. To further confirm that the inhibitory effect of MSE on cell viability is the result of apoptotic cell death, isolated genomic DNA from HepG2 cells treated with 0-1.0 mg/mL MSE for 48 h was electrophoresed in an agarose gel. From the gel, DNA samples were analyzed by observing if DNA ladders are exhibited. Results showed that MSE induced progressive accumulation of fragmented DNA in a concentration-dependent manner. DNA ladder was observed from 0.3 mg/mL MSE concentration and the density increases as the concentration increases (Fig. 4-5). This suggests that the cells underwent apoptosis after MSE treatment. Moreover, there is a good correlation between the extent of apoptosis and the inhibition of cell viability.

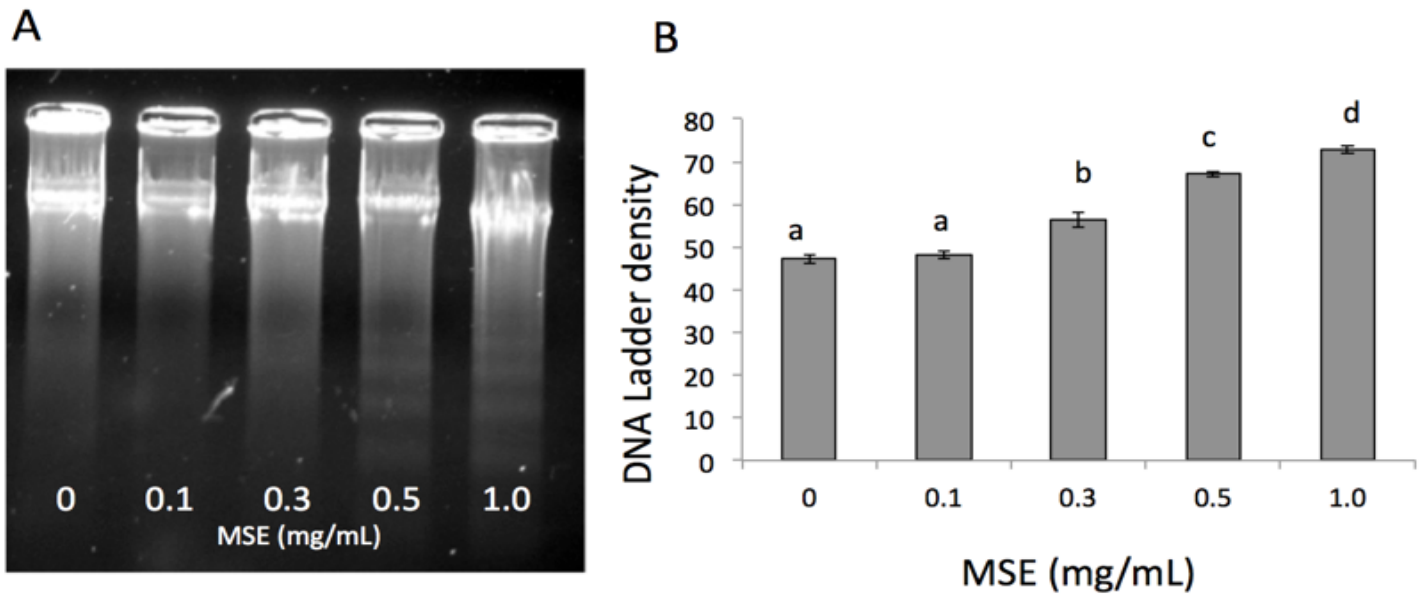


Figure 4-5. Agarose gel electrophoresis of genomic DNA extracted from HepG2 cells treated with MSE for 48 h. (A) DNA ladder analysis (B) Measured DNA ladder density expressed as mean ± SEM of three independent experiments. Columns not sharing the same superscript letters differ significantly (P<0.05).

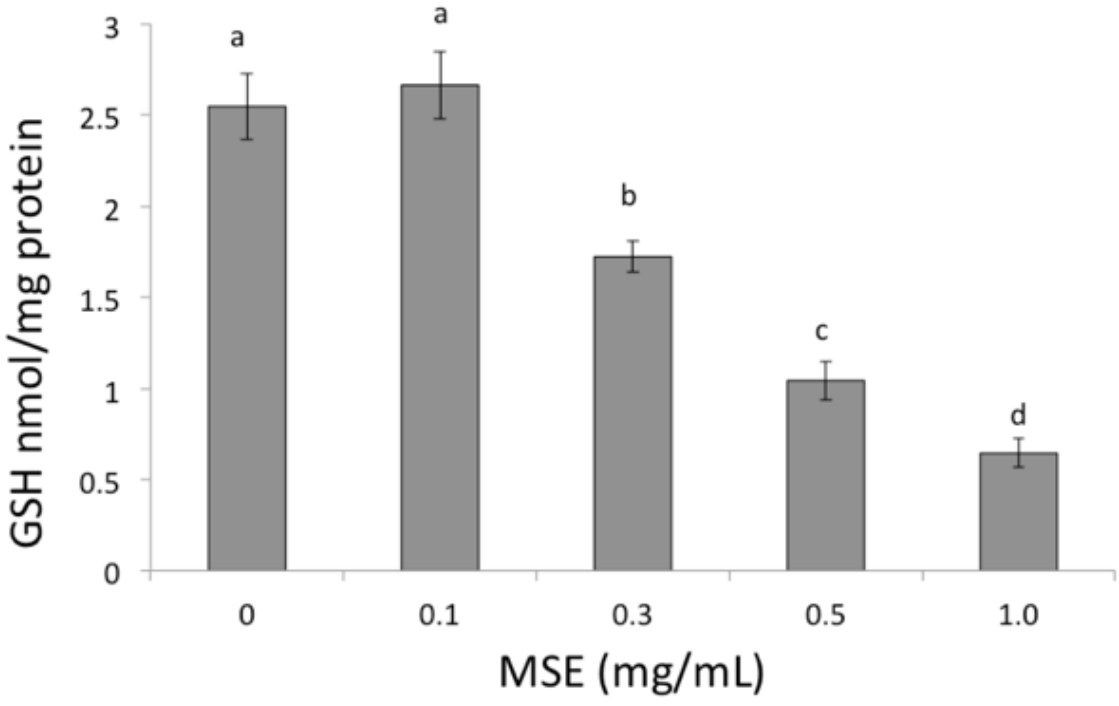


Figure 4-6. Intracellular free-SH levels in HepG2 cells treated with MSE for 48 h determined via DTNB assay. Data are expressed as mean ± SEM of three independent experiments. Columns not sharing the same superscript letters differ significantly (P<0.05).

4.4.5. MSE depletes intracellular free SH levels in HepG2 cells

Glutathione (L-γ-glutamyl-L-cysteinyl-glycine; GSH), the most abundant non-protein thiol in eukaryotic cells, is part of the cell's antioxidant system, which control its redox state. During oxidative stress, GSH is oxidized to GSSG. Thus, it is essential for cell survival and its depletion has been shown either to induce or potentiate apoptosis. (Ortega et al, 2011) Therefore, we determined the intracellular levels of free SH. Fig. 4-6 shows that MSE dose-dependently depletes the intracellular free SH levels. This result suggests that as the concentration of MSE increases, the generation of ROS also increases, leading to the depletion of free SH levels.

4.4.6. MSE inhibits NF-κB activation in HepG2 cells

Since ROS generation has been associated with NF-κB inhibition, the expression of NF-κB p65 and other related proteins on NF-κB pathway were analyzed. As shown in Fig. 4-7, MSE dose-dependently increase the levels of p65 in the cytoplasm after 48 h treatment. Moreover, MSE also induced the dephosphorylation and up-regulation of IkB-α. The accumulation of IkB-α protein signifies the suppression of the proliferation of HepG2 cells. Elevating the levels of IkB-α proteins inhibited the translocation of NF-κB to the nucleus, thereby inhibiting its ability to induce several anti-apoptotic factors. To further confirm this inhibition, we checked the levels of p65 on the nucleus. Results showed that indeed, there was a dose-dependent decrease in the p65 levels in the nucleus, indicating the inhibition in the nuclear translocation of p65.

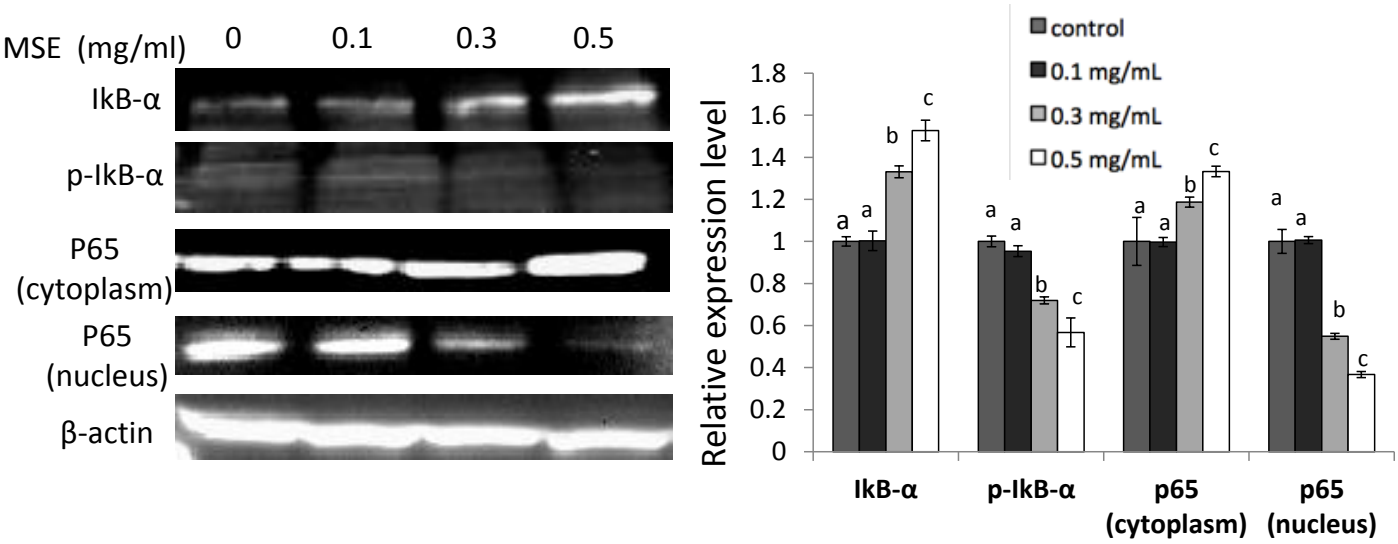


Figure 4-7. Effect of MSE treatment on NF-κB activity. HepG2 cells were treated with different concentrations of MSE and protein extracts were prepared and analyzed by western blotting. Data are expressed as the relative density of each protein to β-actin, mean ± SEM of three independent experiments. Columns not sharing the same superscript letters differ significantly (P<0.05).

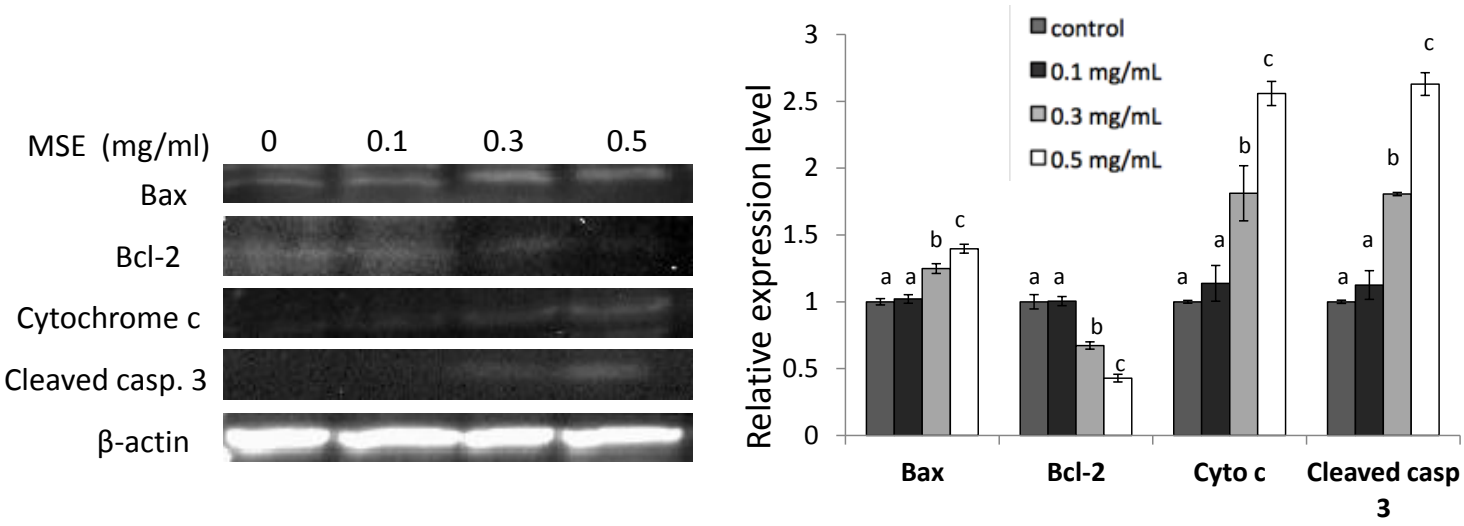


Figure 4-8. Effect of MSE treatment on apoptosis. HepG2 cells were treated with different concentrations of MSE and protein extracts were prepared and analyzed by western blotting. Data are expressed as the relative density of each protein to β-actin, mean ± SEM of three independent experiments. Columns not sharing the same superscript letters differ significantly (P<0.05).

4.4.7. MSE induces mitochondrial apoptotic pathway in HepG2 cells

The results above showed that MSE inhibited HepG2 cell proliferation and induced apoptosis. To determine which pathway is involved in the apoptotic effects of MSE, protein expression of Bax, Bcl-2, cytochrome c and cleaved caspase 3 was measured by western blots after indicated treatments. As shown in Fig. 4-8, after MSE treatment, pro-apoptotic protein Bax, was upregulated while anti-apoptotic Bcl-2 was downregulated significantly. Concomitantly, the release of cytochrome c from mitochondria into cytosol in a dose-dependent manner was observed (Fig. 4-8). The cleavage of caspase 3 is a characteristic feature of apoptosis. Results showed that MSE increases the expression of cleaved caspase 3 dose-dependently. Taken together, these results indicate that MSE induces apoptosis in HepG2 cells via mitochondrial pathway.

4.5 Discussion

In this study, we showed that MSE exerts anti-cancer effects by inhibition of NF-κB activation and induction of apoptosis in hepatocarcinoma cells in vitro. In MTT assay, we observed that the cytotoxic effect of MSE was increasing dose- and time-dependently. Next, we confirmed this cytotoxicity via trypan blue exclusion assay and LDH activity assay. Herein, results showed that indeed, MSE inhibited HepG2 cell proliferation, and caused cell damage in a dose-dependent manner. Annexin V-FITC/PI staining analysis by flow cytometry and internucleosomal DNA fragmentation as determined by agarose gel electrophoresis indicated that the cell death induced by MSE was mediated by apoptosis. A previous study showed that MSE was cytotoxic to several human cancer cell lines (PANC-1, AsPc-1, Pan-02, PC-3,

DU-145, LNCaP, PTEN-CaP8, MCF-7, HT-29 and Colon-26) and induced apoptosis in the cancer cells via caspase 3/7-independent and –dependent mechanisms (Narayanan et al, 2015). However, to our knowledge, there has been no study showing the detailed mechanism or the pattern of caspase activation of MSE in vitro. Our results demonstrated for the first time that MSE decreased cell viability of HepG2 cells through depletion of intracellular free SH levels, inhibition of NF-κB activation and induction of mitochondrial apoptotic pathway.

Apoptosis is an orchestrated cell death, which involves distinct biochemical, and genetic pathways that are responsible for the development and homeostasis in normal tissues (Hassan et al, 2014). The study of apoptosis in diseases is vital as it provides information about the pathogenesis of a disease and at the same time suggests hints on possible ways the disease can be treated (Wong, 2011). Previous researches indicate that insufficient apoptosis can lead to cancer or autoimmunity while enhanced apoptosis is evident in degenerative diseases (Hassan et al, 2014; Brown et al, 2005). When the cell is under various stressful conditions such as precancerous lesions, apoptosis is induced to remove potentially harmful DNA-damaged cells to prevent carcinogenesis (Halazonetis et al, 2008). Apoptosis ensures genomic integrity while its defect initiates the development of cancer. Hence, the ability of malignant cells to evade apoptosis is one of the hallmarks of cancer (Hanahan et al, 2000). The failure of most cancers to proceed to apoptosis in response to apt stimuli is one of the reasons of unsuccessful cancer treatment (Fulda, 2010). As such, there is a greater need for new treatment concepts to rise above cancer resistance to conventional treatment options. Given that natural products such as polyphenols are known to induce apoptotic pathways that are usually

blocked in human cancers, they can have potential therapeutic properties against cancer (Fulda, 2010).

Recent studies have demonstrated that hepatocellular carcinoma cells develop drug resistance primarily by NF-κB activation (Zhao et al, 2014; Omar et al, 2014; Neelgudmath et al, 2015). Thus, the use of cancer therapeutics, which is able to inhibit the activation of NF-κB, and consequently induce apoptosis, will be beneficial for cancer treatment. NF-κB, a protein expressed in nearly all cell types, is a transcription factor that regulates many target genes with various cell functions including inflammation and innate immunity (Hoesel et al, 2013). There are five subunits of this transcription family, identified as p65 (RelA), RelB, c-Rel, p50 and p52, which form a variety of homo- and heterodimers that bind to DNA regulatory sites (Gilmore et al, 2006). Normally, inactive NF-κB dimers are located in the cytoplasm while bound to immunoglobulin kappa light-chain of activated B cells (IκBs). In response to proinflammatory stimuli, IκBα is phosphorylated by IκB kinase (IKK) complex, causing ubiquitin-mediated degradation (Bao et al, 2015). The phosphorylation and degradation of IκB-α, liberated the NF-κB dimers and translocated into the nucleus to activate the transcription of target genes responsible for proliferation, antiapoptosis, drug resistance, angiogenesis and metastasis (Bao et al, 2015; Gilmore et al, 2006). In most cancers including hepatocellular carcinoma, NF-κB is constitutively activated leading to its progression and development (Bao et al, 2015). Here, we found that MSE dose-dependently increased the levels of p65 in the cytoplasm, and induced the dephosphorylation and upregulation of IκB-α. MSE treatment resulted in the dose-dependent accumulation of IκB-α protein, suggesting that MSE suppresses growth in HepG2 cells by elevating the level of IκB-α proteins that inhibits NF-κB possibly by modulating IKK activity. In

addition, MSE inhibited the nuclear translocation of p65 as evident in the dose-dependent decrease of p65 levels in the nucleus.

ROS are known to induce the NF-κB pathway in the cytoplasm, but inhibits NF-κB activity in the nucleus (Morgan et al, 2011; Kabe et al, 2005). Increased intracellular ROS generation causes the depletion of various antioxidant systems that neutralize toxic oxidants such as ROS, and ultimately leads to oxidative stress (Fruehauf et al, 2007). GSH is one of the antioxidant systems that are essential in maintaining the intracellular redox balance. During oxidative stress, GSH undergoes thiol-disulfide exchange producing GSSG, thereby depleting GSH. In order to find out if ROS is involved in the inhibition of NF-κB pathway activation, the intracellular free SH levels were determined. Results showed that MSE depletes the free SH levels dose-dependently which eventually led to the induction of oxidative stress in HepG2 cells (Fig. 7). MSE contains polyphenols such as resveratrol dimer and other resveratrol derivatives. Resveratrol and other polyphenols are known antioxidants and antioxidants exhibit different biological activities in cancer cells and in non-transformed cells (Miki et al, 2012). Antioxidants including resveratrol effectively induced apoptosis in various cancer cells via ROS generation (Miki et al, 2012; Luo et al, 2013, Lang et al, 2015). In the present study, our results suggest that MSE induced ROS generation by depleting the free SH levels in HepG2 cells.

Induction of oxidative stress initiates a cascade of signals which leads to mitochondrial mediated apoptosis. Increase in ROS leads to the free radical attack of the membrane phospholipids in the mitochondria (Li et al, 2006). This causes the loss of the mitochondrial membrane potential, which ultimately leads to the release of intermembrane proteins such as cytochrome c out of the mitochondria and to the cytosol. The release of cytochrome c triggers caspase-3 activation through caspase-9.

Activation of caspase-3, the main executioner of apoptosis, induces DNA fragmentation, nuclear chromatin condensation and eventually, apoptosis (Bras et al, 2005). Bcl-2 family of proteins, which are located in the outer mitochondrial membrane, are also involved in the mitochondrial apoptotic pathway. Pro-apoptotic Bax, when activated, leads to membrane pore formation and permeabilization, promoting the release of cytochrome c into the cytosol (Kudryavtseva et al, 2016). Meanwhile, antiapoptotic Bcl-2 prevents apoptosis via interaction with pro-apototic proteins (Kudryavtseva et al, 2016). In the present study, MSE induced mitochondrial apoptotic pathway by upregulation of pro-apoptotic protein Bax and downregulation of anti-apoptotic protein Bcl-2. This is accompanied by dose-dependent release of cytochrome c followed by the increased expression of cleaved caspase-3. These results, taken together, clearly demonstrate that MSE induces apoptosis via mitochondrial pathway in HepG2 cells. A previous study on MSE-treated HT-29 and PC-3 cells showed that MSE induced extrinsic pathway but not the mitochondrial apoptotic (intrinsic) pathway (Narayanan et al, 2015). Our results, for the first time, demonstrated that MSE could induce apoptosis via the intrinsic/mitochondrial apoptotic pathway in cancer cell.

Polyphenols has been found to induce apoptosis and inhibit NF-κB signaling in various cancer cells. Genistein, a polyphenol abundant in soy, exhibited apoptotic effect via NF-κB inhibition in human colon (LoVo and HT-29) and prostate (PC3) cancer cells (Luo et al, 2014; Li et al, 2002). Epigallocatechin-3-gallate, the main polyphenol in green tea, was reported to induce apoptosis in human prostate carcinoma LNCaP cells via negative regulation of NF-κB pathway and stabilization of p53 (Hastak et al, 2003). Resveratrol dimers and trimers have been found to induce apoptosis in human promyelocytic leukemia cells (HL-60) (Kang et al, 2003). Melinjo

seed extract and its active ingredient gnetin C, a resveratrol dimer, induced apoptosis in pancreatic, prostate, breast and colon cancer cells (Narayanan et al, 2015). Here, we found, for the first time, that MSE induced mitochondria mediated apoptosis and inhibited NF-κB activity through oxidative stress.

4.6. Conclusion

In conclusion, our data provide evidence for the first time that MSE inhibits proliferation and induces mitochondrial apoptosis in HepG2 cells through oxidative stress causing NF-κB inhibition, cytochrome c release and capase-3 activation (see our proposed mechanism as shown in Fig. 4-9). Inhibition of NF-κB represents a novel approach to cancer drug development; thus, MSE may be developed as a potential lead compound or can be used in combination with existing clinical drugs for more effective treatment of hepatocellular carcinoma.

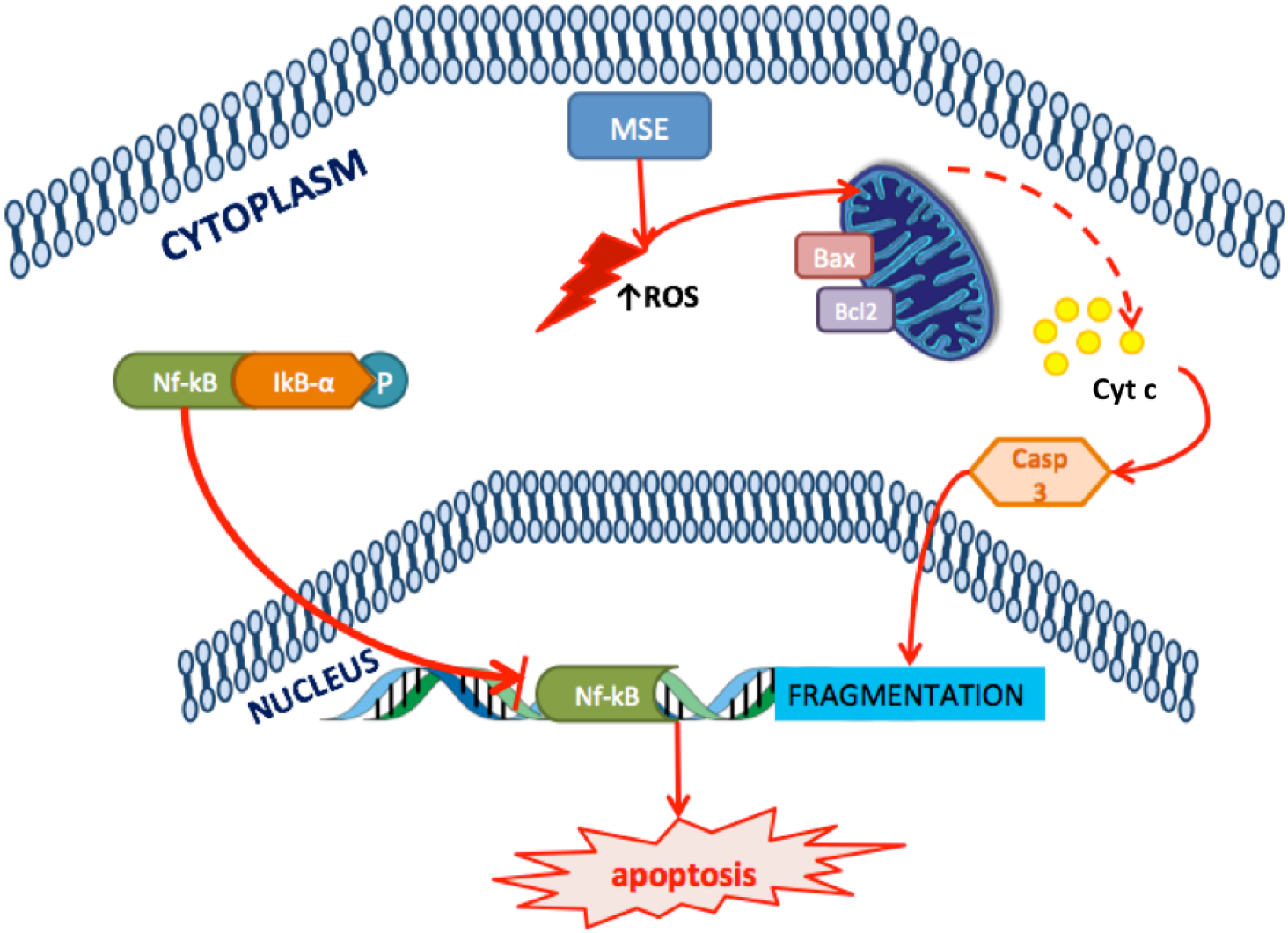


Figure 4-9. Proposed mechanism of MSE-induced apoptosis in HepG2 cells.

4.7. References

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CHAPTER V.

Summary and Conclusion

In this study, the beneficial effects of melinjo seed extracts (MSE) were investigated *in vivo* and *in vitro*. *In vivo* experiments were performed wherein the effects of maternal fructose consumption during pregnancy on the offspring have been studied and the hypothesis that supplementing with MSE can offset these harmful effects has been tested. The key findings of this study are (i) the level of renal pAMPK in FM of 17-week female offspring, but not of the male offspring, was increased after maternal MSE intake during lactation; (ii) maternal fructose intake down-regulated renal eNOS expression in FC group while maternal MSE consumption maintained renal eNOS expression in FM group of female offspring but not of the male offspring; and (iii) maternal MSE intake during lactation lowered the systolic blood pressure in FM of 17-week female offspring, but not of the male offspring. Our data demonstrate that MSE consumption during lactation improved vasodilation and attenuated the development of hypertension in the 17-week female offspring of fructose-fed pregnant rats.

Cancer remains to be one of the leading causes of death worldwide and there is still no drug that is found to be completely effective and safe. Thus, the search for new anti-cancer drugs that will be more selective and have lesser side effects is still a major challenge. Polyphenols from plants display many anticarcinogenic properties including inhibitory effects on cancer cell proliferation, tumor growth, angiogenesis, metastasis, and inflammation as well as inducing apoptosis. In the present study, *in vitro* screening of MSE against a panel of human cancer cells revealed its cytotoxicity against HepG2 liver cancer cells, HeLa cervical cancer cells, OSRC-2 renal cancer cells, and H460 lung cancer cells. Among the four tested human cancer cell lines, HepG2

was found to be the most sensitive to MSE with the IC_{50} value of 171.5 μg/mL. Accordingly, the effect of MSE treatment in HepG2 cells was further investigated. In MTT assay, we found that MSE dose- and time-dependently induced cytotoxicity in HepG2 cells. Annexin V-FITC/PI staining analysis by flow cytometry and DNA ladder analysis indicated that MSE induced apoptosis in a dose-dependent manner. This apoptotic effect of MSE was found to be associated with reactive oxygen species generation suggested by the dose-dependent depletion of intracellular free-SH levels. Western blot data showed the modulation of Bcl-2 family of proteins, cytochrome c release and cleavage of caspase 3 implying the induction of mitochondrial apoptotic pathway. Furthermore, MSE inhibited the activation of the prosurvival NF-κB pathway via induction of dephosphorylation and up-regulation of IkB-α, which ultimately leads to the inhibition of the translocation of NF-κB p65 to the nucleus. These results provide evidence for the first time that MSE has potent anticancer activity against HepG2 cells, thereby, providing basis for future clinical application of MSE in liver cancer cases.

Natural dietary phytochemicals have been widely used in *in vitro, in vivo*, and preclinical studies against various diseases like cancer and hypertension. Despite the increasing interest on synthetic chemistry as a method to discover and develop drugs, the potential of bioactive plant extracts to provide new and novel products for disease treatment and prevention is still huge and should be explored. Generally, the purpose of screening crude plant extracts is to isolate a pure bioactive compound or synthesize and reproduce this bioactive compound. But still, we cannot disregard that there is a potential in developing synergistic plant-based drugs, which may perform differently when utilized independently.

MSE are known to contain resveratrol dimers and a small amount of trans-resveratrol, which have been reported to have several health promoting properties including anti-tumor, anti-cancer and antihypertensive activities. However, it should be noted that it is difficult to determine whether the beneficial effects observed were due to gnetin C, it's glucosides, trans-resveratrol, their metabolites, or by other components of MSE or the combination thereof. Nevertheless, the findings obtained from this research contribute to the increasing biological activities and beneficial effects of MSE. Finally, from these comprehensive data, we can conclude that MSE has a real potential to be an anti-cancer drug substantiated by its inhibition of NF-κB pathway. In addition, MSE consumption during lactation can be beneficial to the offspring of fructose-treated pregnant rats.