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Regulation of Apoptosis Related Factors in Intrinsic Signaling Pathway by Myricetin *in vitro*

(ミリセチンによる内因性シグナル伝達経路のアポトーシス関連
因子の試験管内での調節)

by

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Acknowledgement

Until now, I still clearly remember the first talking with my supervisor, Professor Kurasaki. I have no background knowledge in this field. At that moment, I hesitated. Graduation in three years if it goes smoothly, means I will do research at school or no-salary work for at least three years, perhaps most of my Chinese friends' career will be stable after three years. However, I did not regret because I chose the field which has attracted me to focus on, and will continue my work if possible. Thus, I would like to thanks for my wisdom decision. One of the most important persons who I must appreciate is my supervisor, Professor Kurasaki Masaaki. I can not smoothly complete my Ph.D if there is no his guide and support. I also cannot be more interested in my research if there is no his patience although I know I made you angry for so many times. The persons who I also would like to show my appreciation to are myparents and my wife - Yingjun Qu, for their support and standing by me until now. Another appreciation and thanks for all my labmates especially Rachael Uson Lopez and Md. Mostafizur Rahman for their assistant and guide. Special thanks to Miyako Komori, our gentle kind-hearted experiment assistant and Yongkun Sun for her patient explanation of research background. Finally, thanks all of the people who help me and give me lots of favor in the three years.

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Chapter 1

1.1 General Introduction

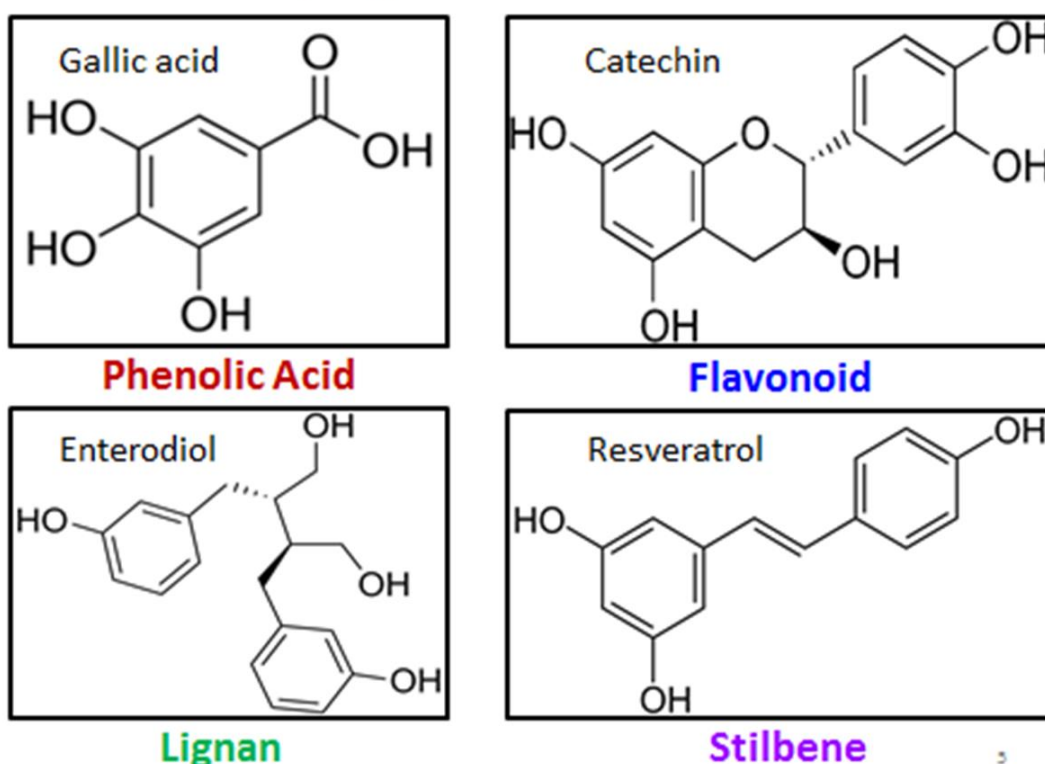
Over the past decades, many researches are focusing on chemotherapy and radiotherapy on cancer. However, despite efforts of many researches, the severe side effects of currently available drugs can not be ignored. As a result, numerous researchers attempt to find, safe and healthy auxiliary compound to satisfy the synergistic effect with some cancer therapies such as chemotherapy, radiotherapy, etc. In the past ten years, dietary supplement manufacturers have shown strong interest in a group of compounds named polyphenols (1). The main reasons why polyphenols have been paid such attention are attributed to their antioxidant properties (2). Polyphenols, as secondary metabolites of plants, are utilized for protection against ultraviolet radiation or aggression by pathogens (3). Naturally, polyphenols are contained in various fruits and vegetables, especially when during the ripeness of plants. For instance, Claudine et al., (2004) (1) pointed out that 100 g fresh weight of fruits such as grapes, apple, pear, cherries and berries contain up to 200-300 mg of polyphenols, while a glass of wine or a cup of tea contains around 100 mg of polyphenols. Over 8000 polyphenolic compounds have been identified in different kinds of plant species. According to the function of phenol rings, the distinction of polyphenols can be classified as phenolic acids, flavonoids, stilbenes, and lignans (1). Phenolic acids can be divided into two classes including benzoic acid and derivatives of cinnamic acid. Gallic acid, as a kind of hydroxybenzoic acid, which is contained in tea leaves up to 4.5 g/kg of fresh weight tea (4). Although the contents of hydroxybenzoic acids are numerous in food, the hydroxycinnamic acids are relatively more common than hydroxybenzoic acids (5). Flavonoids in fruits and vegetables especially quercetin, kaempferol and myricetin can be widely found even though their concentrations are as low as about 15-30 mg/kg of fresh weight. Onions is well known as the richest sources for quercetin, which contains up to 1.2g/kg fresh weight of flavonols (1). In opposite, the flavones are not as common substance as

compared with flavonols. Stilbenes are another type of polyphenol and one of the most well studied stilbene is resveratrol. Moreover, linseed, as a representative of lignans, is the richest dietary source (5).

Antioxidant Effect

Polyphenols commonly exhibited its potent antioxidant property in various aspects such as cardio-protective effect, neuro-protective, anti-diabetes, anti-virus, anti-bacterial and anti-inflammatory.

Distribution of polyphenols



Cardio-protection

Many researchers have demonstrated that consumption of polyphenols improve heart diseases. Renaud et al., (6) has already reported that wine can decrease incidence of coronary heart disease. Another report by Dubick et al., (7) showed that the polyphenols in wine and tea played as modulators of atherosclerosis and ischemic heart disease in humans. The review written by Nardini et al., (8) indicated the roles of dietary polyphenols in platelet aggregation. In detail, quercetin, an abundant polyphenol of onion was utilized in patients with ischemic heart disease (IHD) having metabolic syndrome. As a result, quercetin did not only show

antiischemic and, antiarrhythmic effects, but also exhibited regulating effects on vegetative homeostasis, oxidant disturbances in patients with IHD (9). García-Lafuente et al., (10) reported that flavonoids, which serve as anti-inflammatory agents, can remit the symptoms of coronary heart disease by inhibiting the expression of metalloproteinase 1 (MMP1). Maeda K et al., (11) found that catechins could inhibit invasion and proliferation of the smooth muscle cells in the arterial wall that would be a mechanism, which may contribute to slow down the formation of the atheromatous lesion. Moreover, IHD, which is a leading cause of cardiac dysfunction and subsequent morbidity in the world, is characterized by impaired blood flow to a region of the heart leading to cardiac cell death. The most common manifestation of IHD which can lead to cardiac cell death is heart attack. Past studies have reported that resveratrol exhibited cardioprotection by protecting deleterious effects on the heart in the management in IHD (12). Raj et al., (13) have also discussed the efficacy of resveratrol on cardioprotection. In addition, many studies have been reported that catechin can improve the heart failure. For example, Zhang et al., (14) have investigated that the therapeutic effect of (-)-epigallocatechin-3-gallate (EGCG) on heart failure by inhibiting transfer membrane of GRK2 and reducing desensitization of β 1-AR. Dong et al., (15) demonstrated that EPI (exocrine pancreatic insufficiency) suppressed AngII-induced cardiac hypertrophy by activating the SP1/SIRT1 signaling pathway. Oyama et al., (16) also showed green tea catechin attenuated the progression of heart failure induced by the heart/muscle-specific deletion of MnSOD in mice. Moreover, Peters et al., (17) also implied that the consumption of polyphenol rich diet has been associated to a lower risk of myocardial infarction in both case-control and cohort studies.

Anti-diabetes

Diabetes can be divided into two main categories: type-1 and type-2. Rizvi et al., (18) (19) have shown that several physiological parameters of the body get altered in the diabetic conditions. Long-term effect of diabetes can lead to some progressive development of specific complements such as nephropathy and retinopathy. Previous studies have already shown anti-diabetic potential of polyphenols. Rizvi et

al., (20) investigated the protective role of tea catechins against oxidation-induced damage of type-2 diabetic erythrocytes. Sahebkar et al., (21) reported the effect of curcuminoids plus piperine-modulated adipokines in type 2 diabetes mellitus. The results showed curcumin supplementation increased adiponectin, while the leptin levels were decreased and reflected a decrease in the inflammatory TNF- α level. Zheng et al., (22) reported that the polyphenol rich extract from *Phellinus igniarius* possessed potential anti-diabetic effects including improving glucose tolerance, reducing hyperglycemia, and normalizing insulin levels by the activation of GLUT4 translocation via the modulation of the AMPK pathway. Another polyphenol-rich naturally occurring compound named ethyl acetate fraction (EAF), which is isolated from *Molineria latifolia* rhizome, was utilized as dietary interventions for type 2 diabetes mellitus and its underlying molecular mechanisms *in vivo* (23). It was suggested that EAF exhibited its effect by modulating insulin signaling, potentially via IRS1/AKT activation. The pharmacological attributes of EAF may implicate its potential therapeutic applications for diabetes management (23). Some researchers also tried to use resveratrol to decrease insulin secretion and delay the onset of insulin resistance. Perhaps the mechanism depends on the inhibition of K⁺ + ATP and K⁺ + V channel in β -cells (24). It is clearly known that quercetin a dominant polyphenol of onion, has already exhibited its protective effects on the alterations in diabetic patients by significantly protecting the lipid peroxidation and inhibiting antioxidant system in diabetics (25). Another polyphenol that is abundant in maize bran, is regarded as a potent anti-diabetic agent by lowering blood glucose followed by significantly increased plasma insulin and a negative correlation between blood glucose and plasma insulin (26, 27). Many other studies also showed that polyphenols exhibit potential in relieving the symptoms of diabetes by acting in different levels including inhibiting intestinal glycosidases and glucose transporter (28), decreasing of S-Glut-1 mediated intestinal transport of glucose (29), delaying the transfer of glucose from stomach to the small intestine and modulating SIRT1 by improving whole-body glucose homeostasis and insulin sensitivity in diabetic rats (30, 31).

Anti-inflammatory

Inflammation, a process by which white blood cells attempt to combat injury or foreign organisms such as virus, bacteria or other pathogens, is a kind of immune defense mechanism. It is always accompanied by some symptoms such as redness, swelling, pain, stiffness and loss of joint function. Large amounts of anti-inflammatory drugs have been already widely used such as aspirin, diclofenac, ibuprofen, acetaminophen, and paracetamol. However, unexpected side effects commonly occurred after using medicines. Thus, some researchers tried to develop safer compounds and mechanism-based approaches for the management of diseases. Polyphenols have been proved in recent several decades as anti-inflammatory agents. Yoon et al., (32) focused on some pivotal molecular targets of polyphenols that directly affect the inflammation process. Joseph et al., (33) made a summary providing a comprehensive overview of human clinical trials which investigated the acute and chronic effects of polyphenols from commonly consumed fruits or their derived products on inflammation. Additionally, some researchers utilized a polyphenols-rich extract from tea (*Camellia sinensis*) flowers to treat inflammation in acute and chronic mice models (34). Santos et al., (35) used a natural polyphenol, chlorogenic acid to evaluate the anti-inflammatory, analgesic and antipyretic activities. Some researchers summarized the effects of flavonoids and other polyphenols on inflammation (36). Recently, some novel studies showed flavonoids exhibit its anti-inflammatory potential in neurodegenerative disorders (37). They found pure flavonoids or enriched-extracts function as anti-inflammatory agents by reducing the expression of pro-inflammatory cytokines (IL-6, TNF- α , IL-1 β and COX-2), down-regulating inflammatory markers and preventing neural damage (37). This effect is mainly related to the regulation of microglial cells, mediated by MAPKs and NF- κ B signalling pathways on the basis of their *in vitro* and *in vivo* experimental results. It was implied the role of inflammation in neurodegenerative diseases, and the potential therapeutic effects of flavonoids as a promising approach to develop innovative neuroprotective strategy.

Prooxidant Effect

Perhaps most of researchers paid attention about anti-cancer effects of polyphenols because their pro-oxidant property. And the pro-oxidant effect plays the most important role in the mechanism of cancer protective process by inducing reactive oxidative stress. Various studies have reported that polyphenols exhibit its potential for cancer therapy reagents as major or auxiliary therapeutic drug with chemotherapy or radiotherapy. The anti-cancer effect can be realized by reducing the numbers and growth of tumors (38). Numerous polyphenols showed their protective effect in some models even though their mechanisms of action were found to be different (39). García-Lafuente et al., (40) have identified for chemoprevention effects of polyphenols by mechanisms of action including estrogenic/anti-estrogenic activity, anti-proliferation, formation of detoxification enzymes, regulation of the host immune system, anti-inflammatory activity and changes in cellular signaling. Past studies have demonstrated the combined effects of polyphenols with other anticancer drugs or therapies. For example, Lewandowska et al., (41) reported synergistic interactions between anticancer chemotherapeutics and phenolic compounds. Additionally, Fantini et al., (42) reported that the anticancer effects of combinations of polyphenols or polyphenols and anticancer drugs, with a focus on their ability to modulate multiple signaling transduction pathways involved in cancer. A review focusing on the dietary polyphenols and prevention of diseases showed that polyphenols modulate the expression of cytochrome P450 enzymes involved in the activation to carcinogens so as to influence the metabolism of pro-carcinogens (43). Potentially toxic quinones which are substrates of these enzymes can be formed by polyphenols. In addition, these enzymes can be activated by the intake of polyphenols for their detoxication to induce a general boosting of our defenses against toxic xenobiotics (44). The cancer preventive activity was shown by administration of polyphenol to inhibit the conversion of high grade prostate intraepithelial neoplasia (PIN) to men having high-grade PIN (45). Another polyphenol named as myricetin also exhibited anticancer on human anaplastic thyroid cancer via mitochondria dysfunction (46). Moreover, myricetin increased the cytotoxicity of paclitaxel due to the significant down-regulation of MDR-1 in these

cells when it was used for pre-incubation of ovarian cancer cells in lower dose manner (47). Yang et al., (48) also proved that myricetin suppresses invasion and promotes cell death in human placental choriocarcinoma cells through induction of oxidative stress. In addition, quercetin does not only possess anticancer property against benzopyrene induced lung carcinogenesis in mice, but also its free radical scavenging activity (49). These studies provided a potent proof for supporting the use of dietary polyphenols in human cancer chemoprevention, in a combinational approach with either chemotherapeutic drugs or cytotoxic factors for efficient treatment of drug refractory tumor cells. Polyphenols often show anti-carcinogenic effects by the induction of apoptosis mediated by signaling pathways especially mitochondrial or intrinsic signaling pathway to protect all stages of development of cancer. Mitochondria, which plays a critical role in apoptosis, is closely associated with some death signals and trigger apoptosis by releasing death factors into cytosol, such as cytochrome c. Thus, in the following study, one kind of commonly natural occurring polyphenol named myricetin is utilized for clarifying its prooxidant property mediated by intrinsic signaling pathway.

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Chapter 2

Myricetin enhances apoptosis induced by serum deprivation in PC12 cells mediated by mitochondrial signaling pathway

2.1 Abstract

Polyphenols have many beneficial effects and an effective disease therapeutic auxiliary drug. Previously, myricetin, a polyphenol, had been reported to possess various biological effects on human physiology. However, mechanism of myricetin on apoptosis induced in PC12 cells is still unclear. PC12 cells were treated with myricetin in two concentration levels comprising 0.1 and 1 μ M under serum-free condition. As a result, morphological changes were observed using trypan blue assay. DNA fragmentation was determined by DNA ladder assay to evaluate DNA damage levels. Western blotting results showed that cytosolic cytochrome c which was released from mitochondria. Subsequently, tumor suppressor gene p53, pro-apoptotic and anti-apoptotic Bcl-2 family proteins Bax and Bcl-2 were expressed. The caspase cascade reaction was induced through caspase 3 and 9 expression. It is predictable that serum deprivation as a stimuli activated tumor suppressor gene p53. The activation of p53 induced the transcriptional activation of Bax versus Bcl-2 dimer and then induced release of mitochondrial cytochrome c into cytosol. Cytosolic cytochrome c, binds with Apaf-1, forms a complex with procaspase-9 to activate caspase-3. It is confirmed that the occurrence of apoptosis from DNA fragmentation. It is suggested that myricetin significantly enhanced the apoptosis induced by serum deprivation in a dose-dependent manner in PC12 cells.

Keywords

Polyphenol; Apoptosis; Serum deprivation; Protein expression; Mitochondria; Apoptotic signaling pathway

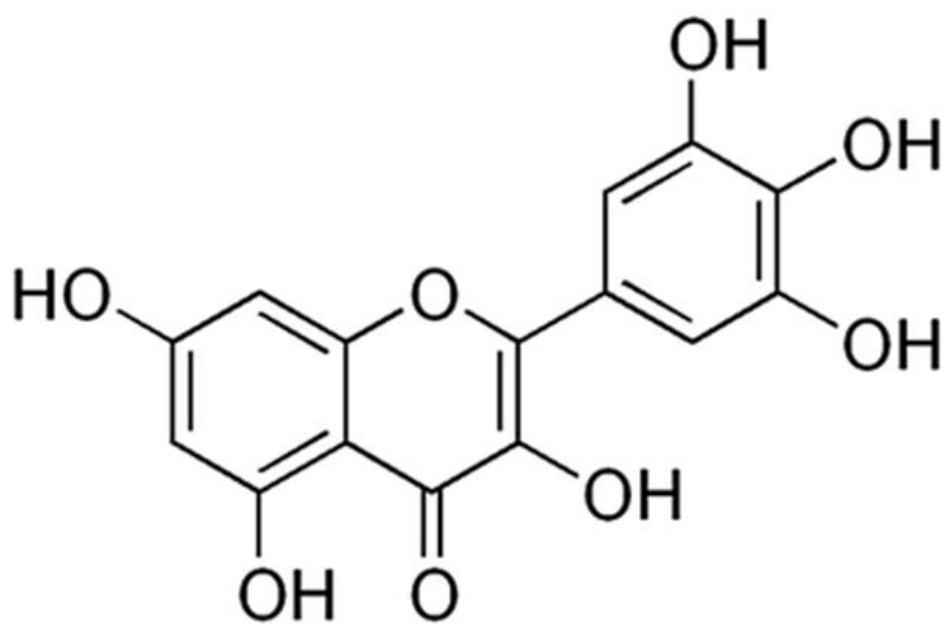
2.2. Introduction

Currently, radiation therapy is the most commonly used cancer treatment despite its known irreversible adverse effects (Bentzen, 2006). In addition, intravenous anticancer drugs such as doxorubicin, paclitaxel, and cisplatin also exhibit severe adverse effects (Aluise et al., 2010; Esmaeli and Valero, 2013; Florea and Büsselberg, 2011) including cardiomyopathy, alopecia, vomiting and hematochezia. Thus, the search for cancer treatments is shifting to potent naturally original chemical substance like polyphenols, which serve as supplements to exhibit no severe adverse effects.

Polyphenols are rich micronutrients in our daily diet and researches revealing their role in the protection from degenerative diseases like cancer are emerging (Manach et al., 2004). Quercetin, is a polyphenol that exhibited inhibitory effect on colorectal lung metastasis through induction of apoptosis, and suppression on metastasis (Kee et al., 2016). Green tea polyphenols inhibited proliferation and induced apoptosis on HL-60 cells (Han et al., 2009).

Myricetin (3, 5, 7, 3', 4', 5'-hexahydroxy flavone) is a polyphenol that is abundantly found in fruits like berries such as blueberries and strawberries (Hertog et al., 1993; Häkkinen et al., 1999). Previous studies show its critical role of pharmacologically biological activities on various fields. It can act as an antioxidant by scavenging free radicals which exist in the both enzymatic and non-enzymatic systems. It was also regarded pro-oxidant by damaging carbohydrates and DNA. It can enhance DNA degradation in vitro because of the presence of anti-oxidant Fe^{3+} and Cu^{2+} . Myricetin can also be a mutagen (MacGregor and Jurd, 1978; Uyeta et al., 1981). Myricetin exhibited anti-viral effect especially as an inhibitor of reverse transcription (Ono and Nakane, 1990). It can inhibit DNA/RNA replication and repair by inhibiting DNA polymerases (Ono and Nakane, 1990). Interestingly, myricetin also showed anti-aggregatory effects on blood platelets both in vitro and in vivo (Landolfi et al., 1984; Robak et al., 1988), anti-atherosclerotic lesions by inhibiting oxidative modification of LDL (de Whalley et al., 1990) and anti-diabetic by stimulating

lipogenesis and enhance insulin stimulated lipogenesis in isolated rat adipocytes (Ong and Khoo, 1996).



Myricetin



Myricetin is abundant especially in most of berries

Apoptosis, a process of programmed cell death, is a cellular evolutionary mechanism to remove damaged, misplaced and redundant cells in order to maintain tissue homeostasis and development (Alvarez-Tejado et al., 2001). There are several ways in which apoptosis can be induced by: (1) absence of neurotrophins; (2) ultraviolet radiation or γ -radiation; and (3) chemotherapy drugs. During apoptosis, a series of morphological changes such as cell membrane reversal, translocation of phosphatidylserine residues, blebbing, nuclear fragmentation, and chromatin condensation, are induced. In this process, it simultaneously accompanies DNA damage after related genes expression and cleavage of death substrates as the last puzzle to accomplish apoptosis (Elmore, 2007).

Mitochondria is an important chemical-energy output source which can induce oxidative phosphorylation and adenosine triphosphate (ATP) production. In apoptosis, it plays a central role in sensitizing cellular death signals by releasing mitochondrial cytochrome c into cytosol. The release of cytochrome c is directly related with the expression of apoptotic related Bcl-2 family genes. Bax gene, as a pro-apoptotic homologue of Bcl-2, interacts with Bcl-2 which is a member of anti-apoptotic factors in Bcl-2 family genes and then forms a kind of heterodimer to increase the opening of mitochondrial voltage-dependent anion channel (Jürgensmeier et al., 1998). This results to loss of mitochondrial outer membrane potential and damage on the permeability transfer pores. Mitochondrial cytochrome c, which released into cytoplasm, binds with Apaf-1 and then forms a complex with procaspase 9 to induce the activation of down-stream caspase 3. Subsequently, the death substrate such as PARP is cleaved. On the other hand, tumor suppressor p53 gene can trigger apoptosis (Chipuk et al., 2004) through the induction of the transcription of Bax and influencing the ratio of Bax versus Bcl-2 protein level (Hemann and Lowe, 2006).

It was reported that myricetin could induce apoptosis of human leukemia (HL-60) cells via mitochondrial pathway, but not the ROS dependent pathway (Ko et al., 2005). Myricetin has been reported to exhibit antiproliferative effects in human glioma U251 cells (Li et al., 2016). Another study on the induction of apoptosis by myricetin

on cancerous hepatocytes by targeting their mitochondria has been reported (Seydi et al., 2016). Moreover, myricetin also showed its inhibitory potential on proliferation of cisplatin-resistant ovarian cancer cell lines OVCAR-3 and A2780/CP70 through a p53-dependent apoptotic pathway (Huang et al., 2015). While the effects of myricetin in apoptosis on other cell lines have been studied, the effects of myricetin during serum deprivation induced apoptosis in PC12 cells are still unclear. We hypothesized that if apoptosis will be enhanced, the expression of some related apoptotic genes such as p53, Bax, Bcl-2, cytochrome c and proteins in the caspase cascade reaction are expected to be promoted by myricetin.

2.3. Materials and methods

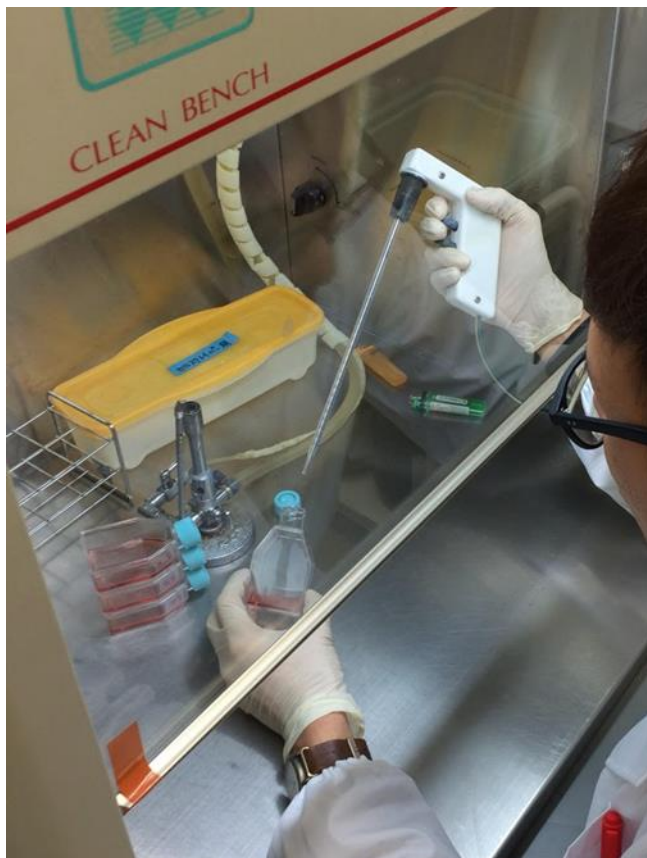
2.3.1. Chemicals

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM) was bought from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from HyClone (Rockville, MD, USA). Ribonuclease A (RNase), ethidium bromide, and peroxidase-conjugated avidin were purchased from Sigma (St. Louis, MO, USA). Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Biotinylated donkey anti-rabbit immunoglobulin was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Polyclonal antibodies against Bcl-2 (Merk-Millipore; Darmstadt, Germany), Bax (Bioss Antibodies; Woburn, MA, USA), beta-actin (GeneTEX; Irvine, CA, USA) were purchased. Anti-caspases 3 and 9 were purchased from BD Biosciences (Franklin Lakes, NJ, USA) and GeneTex (Irvine, CA, USA). Anti-p53 was bought from Cell Signaling Technology (Beverly, MA, USA). Anti-cytochrome c monoclonal antibody was purchased from Pharmingen (San Jose, CA, USA). Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). The DNA 7500 assay kits were purchased from Agilent Technologies (Waldbronn, Germany). Myricetin, was purchased from Tokyo Chemical Industry Company (Tokyo, Japan). All other chemicals were of analytical grade.

2.3.2. Cell culture

PC12 cells were cultured in a humidified incubator at 37 °C and 5% CO₂ with 10% of FBS 24 h prior to treatment. Myricetin was diluted with ethanol into two concentration levels of 0.1 and 1 mM. Two groups including control (10% FBS) and serum free medium FBS (–) were performed prior to myricetin treatment in order to confirm whether apoptosis will occur under serum deprived condition. Thereafter, three groups were set including serum free medium FBS(–), 0.1 μM (5 mL of

serum-free medium with 5 μ L of 0.1 mM myricetin) and 1 μ M (5 mL of serum-free medium with 5 μ L of 1 mM myricetin). These administration amounts were decided on the basis of daily intake amounts of myricetin explained by Jayakumar et al. (2014), and Thilakarathna and Rupasinghe (2013). The treatment period lasted for 72 h.



2.3.3. Cell viability

Trypan blue staining assay was used to determine the cell viability following the protocol which previously described by Rahman et al. (2017). PC12 cells were seeded at a density of 1×10^6 cells/flask and preincubated for 24 h. After 72 h, the cells were collected and then stained in 0.2% trypan blue solution in phosphate-buffered saline (PBS) solution. Dead cells were stained into blue color. Morphologic changes were detected by microscope observation, and cell numbers were measured by hemocytometer (TC10™ Automated Cell Counter, Bio-Rad).

2.3.4. DNA extraction

The cells were washed with PBS after harvesting. Genomic DNA was isolated with High Pure PCR Template Preparation Kit (Roche Life Science; Penzberg, Germany) following the manufacturer's instructions. Ethanol precipitation method was used to recover DNA. The DNA ladder pattern was quantified using agarose gel electrophoresis. After electrophoresis, the gel was soaked in ethidium bromide solution for 15 min in the dark, and DNA was visualized and photographed under UV illumination with ChemiDoc XRS (Bio-Rad) to determine the extent of cell death. The density level of DNA ladder was estimated by Image J software.

2.3.5. Western blot analyses of apoptotic gene expression

Cytosolic cytochrome c released from mitochondria was quantified using cytochrome c release apoptosis assay kit (Merk-Millipore, Darmstadt, Germany). After 72 h treatment, the cells were harvested and suspended in 1 mL of ice-cold PBS. After removal of the supernatants by centrifugation, the cells were resuspended in 80–120 μ L of cytosol extraction buffer mix (1Xcytosol extraction buffer containing 1 mL of protease inhibitor cocktail and 2 mmol/L DTT). The mixture was allowed to stand on ice for 10 min, and subsequently the cells were disrupted by sonication for 30 s using a Sonicator 250 (Branson). To remove the unbroken cells, the lysed cells were centrifuged at 3000 rpm for 10–15 min. The supernatant was transferred into a 1.5 mL tube and centrifuged at 10,000 rpm for 30 min. The obtained final supernatant was collected as the cytosolic fraction for cytochrome c analysis.

For determination of β -actin, Bax, Bcl-2, p53, caspases-3 and -9, lysis buffer (100 mmol/L HEPES, 1 mol/L NaCl, 300 mmol/L EGTA, 0.1 M PMSF, 100 mmol/L Na_3VO_4 , 10 mmol/L Na_2MgO_4 , 100 mmol/L 2-glycerophosphoric acid, 1 mmol/L MgCl_2 , 100 mmol/L DTT, 100 mmol/L NaF, and triton X-100) was used to collect the protein fraction after two cycles of sonication. Thereafter, the approximately equal content of protein was applied and separated on 12.5% polyacrylamide gel, and the electrophoresed proteins were transferred to nitrocellulose membranes with a

semidry blotting system, type AE6678 (ATTO, Tokyo, Japan). The membranes were incubated overnight at 4 °C in 5% skim milk as a blocking agent. The membranes were incubated for 1 h at 37 °C with the primary antibodies, washed three times, and then incubated with the secondary antibody for 1 h. After antibody reaction, related protein expression was imaged in the way of protein bands with an enhanced chemiluminescence imaging system (ChemiDoc XRS, Bio-Rad). The band density level was measured with Image J software.

2.3.6. Statistical analysis

All the experiments were performed at least three times. The data were analyzed for significant difference using Student's t-test and one-way ANOVA. P values ≤ 0.05 were considered to indicate statistically significant differences. The mean $\pm$ SD values were calculated for all variables.

2.4. Results

2.4.1. Enhancement of myricetin on cell proliferation induced by serum deprivation in PC12 cells

In the present study, cell viability was determined to confirm whether myricetin can inhibit growth and proliferation of PC12 cells. PC12 cells were treated with increasing levels of concentration of myricetin for 72 h. After 72 h, we found that indeed, there is no significant difference under normal condition (10% FBS) (Fig. 1) and serum deprivation induced apoptosis and that almost 60% of cells died since no nutritional factors were supplied to the cells (Fig. 2). Moreover, it was found that myricetin enhanced the apoptosis induced by serum deprivation with dose increasing. Morphological changes were detected through microscope observation. As the concentration increases, more cell shrinkage and volume reduction were induced.

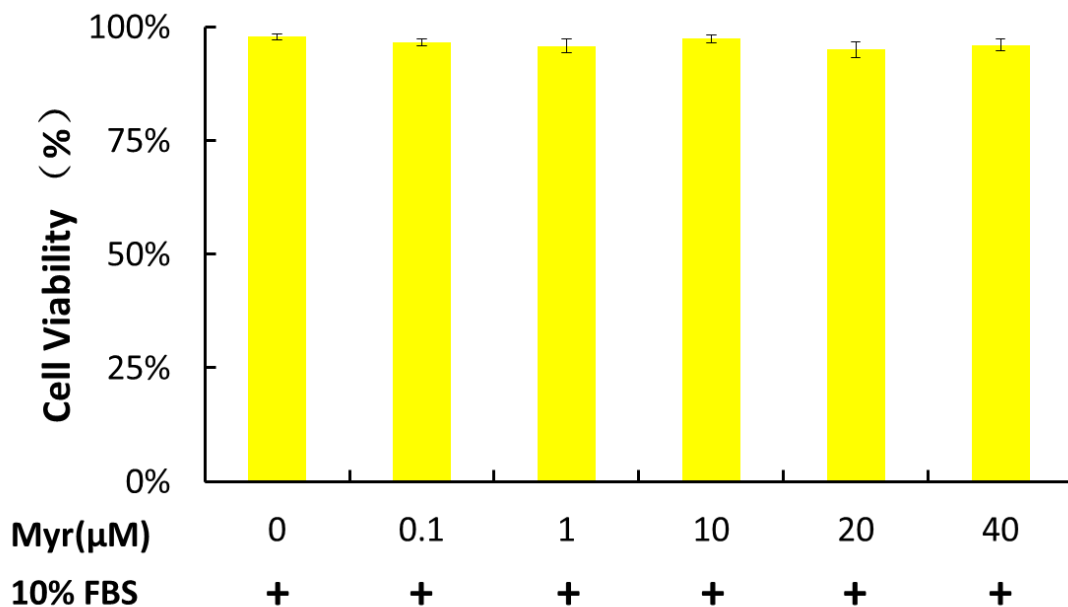


Fig. 1. After 72 h, cell viability of PC12 cells was determined by trypan blue staining in 10% FBS medium containing 0, 0.1, 1, 10, 20 and 40 μM myricetin. Error bars indicate SEM (n = 3).

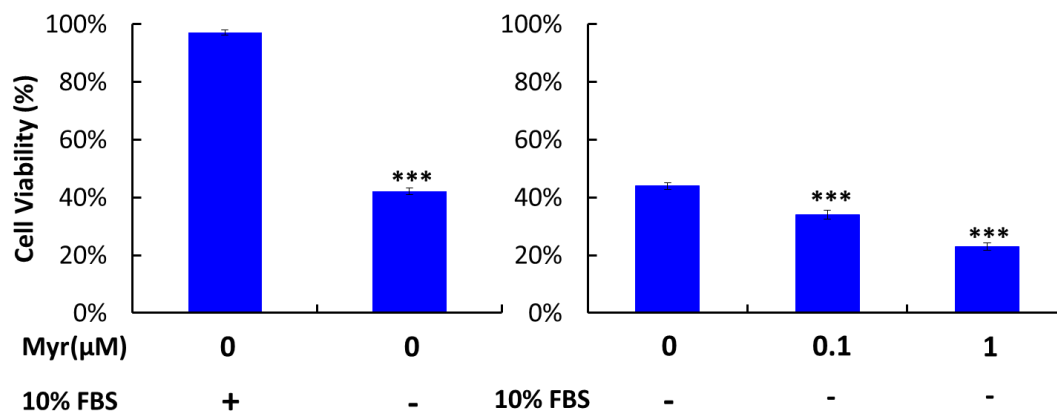


Fig. 2. After 72 h, cell viabilities of PC12 cells were determined by trypan blue staining in 0 and 10% FBS medium, and in FBS-free medium containing 0, 0.1 and 1 μ M myricetin myricetin. Error bars indicate SEM (n = 5). *** indicates significant differences at $p < .01$ and $p < .001$, respectively.

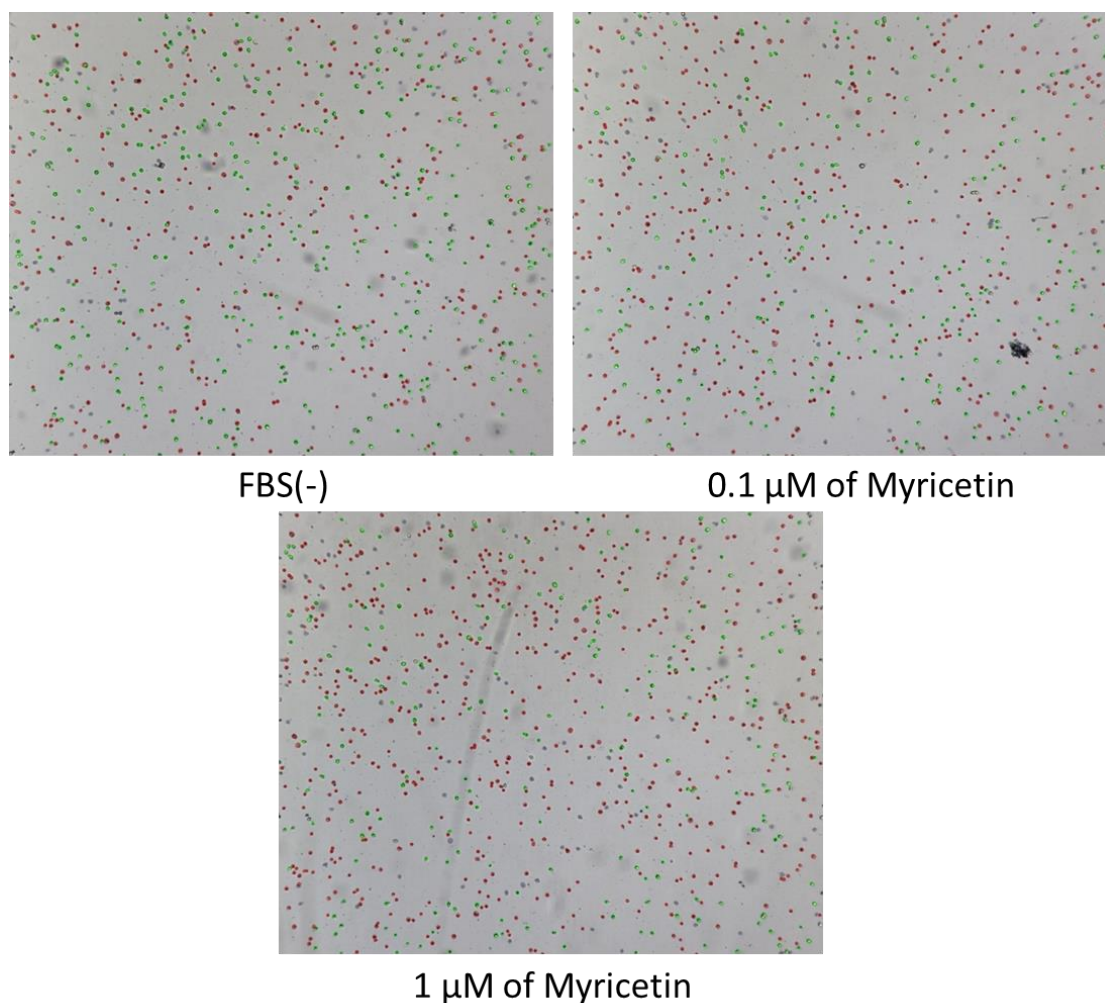


Fig. 3. After 72 h, cell viabilities of PC12 cells were determined once by trypan blue

staining in serum-free medium containing 0, 0.1 and 1 μM of myricetin for cell morphocology observation.

2.4.2. DNA fragmentation

The extent of DNA damage was observed through DNA ladder assay under UV illumination. Clear band fragmentation could be observed after serum deprivation compared with the control group (Fig. 2A). This confirmed that indeed, serum-free treatment induces apoptosis. Furthermore, higher density of band fragmentation was observed after myricetin treatment in a dose-dependent manner (Fig. 2B).

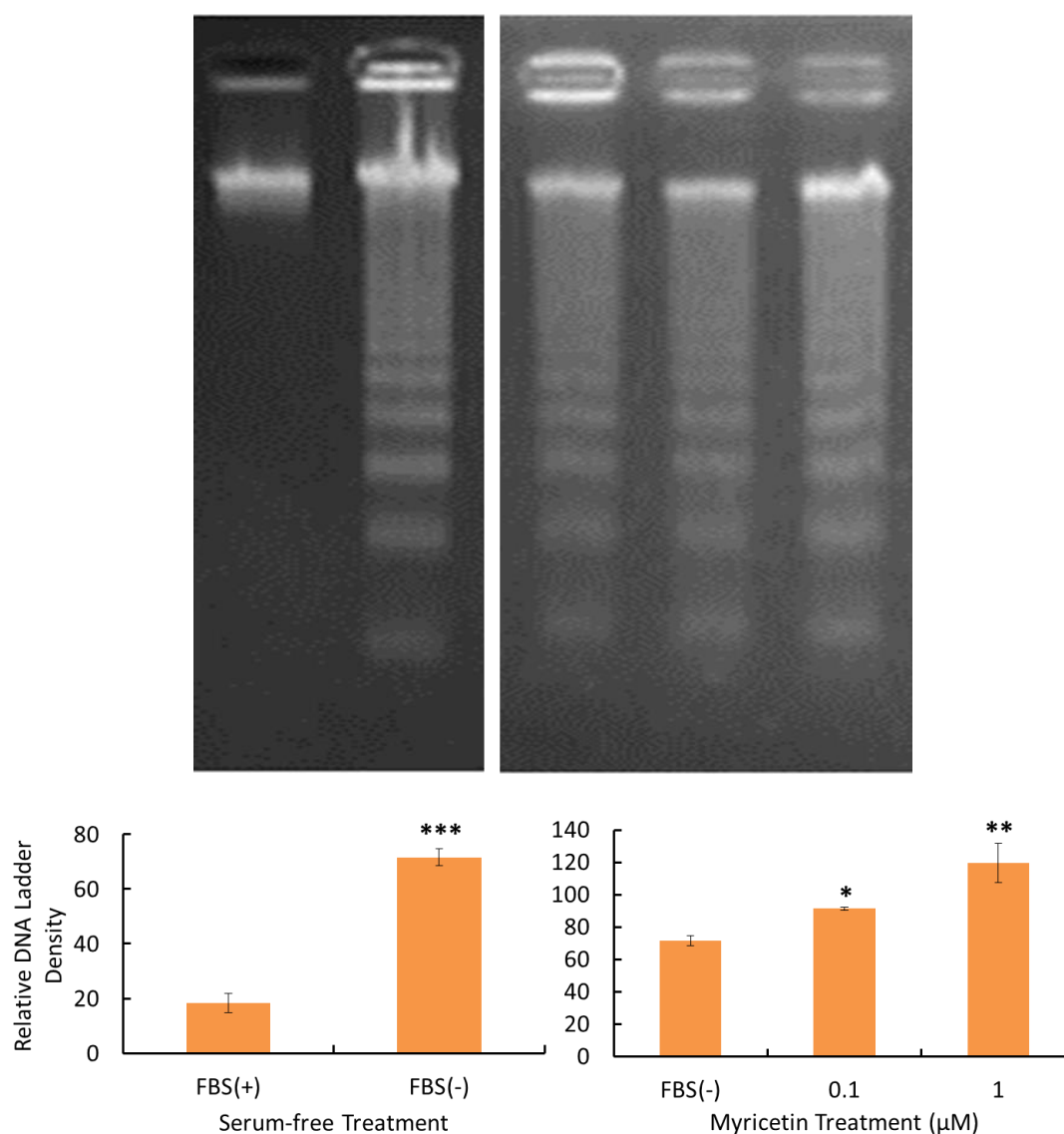


Fig. 3. DNA fragmentation was evaluated by DNA ladder assay in PC12 cells treated

with 0 and 10% FBS (left) and with 0, 0.1 and 1 μ M myricetin under serum deprivation condition (right) after 72 h. Error bars indicate SEM (n = 5). *, ** and *** indicate significant differences at p < 0.05 0.01 and 0.001, respectively.

2.4.3. Mitochondrial cytochrome c released into cytosol

Previously, it has been reported that cytochrome c was released from the mitochondria into the cytosol during apoptosis (Gogvadze and Orrenius, 2006; Orrenius et al., 2007). In the present study, we followed the tracks of cytochrome c in the cytosol using western blot assay. Indeed, mitochondrial cytochrome c was expressed in the cytosol due to poor nutrition supply during serum deprivation (Fig. 3A). In addition, increased levels of cytochrome c were detected in the myricetin-treated groups following a dose-dependent manner (Fig. 3B).

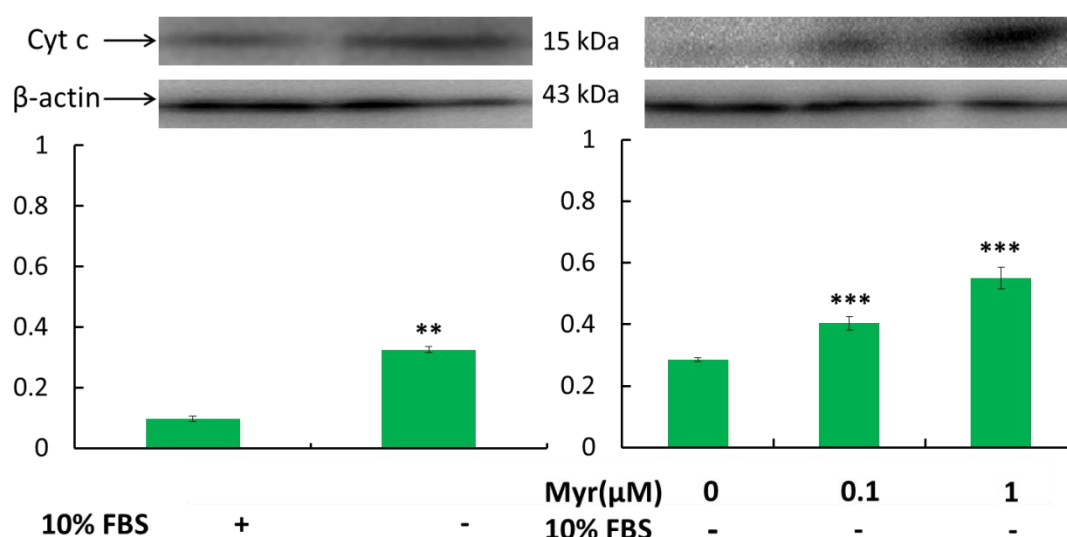


Fig. 3. Cytosolic cytochrome c contents in PC12 cells treated with 0 and 10% FBS (A) and with 0, 0.1 and 1 μ M/mL myricetin under serum deprivation condition (B) for 72 h were determined by western blot. Error bars indicate SEM (n $\geq$ 3). * and *** indicate significant differences at p < .05 and p < .001, respectively.

2.4.4. Expression of pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2

With dose increase, higher levels of mitochondrial cytochrome c were stimulated by myricetin and subsequently released to cytosol. Jürgensmeier et al.

(1998) has indicated that pro-apoptotic Bax can directly induce release of cytochrome c from isolated mitochondria through forming an iron channel with anti-apoptotic Bcl-2 to induce the loss of mitochondrial membrane potential. Further experiment was essential in order to clarify cytochrome c release mechanism. Thus, the expression of pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 were determined. Bax expression tended to be a positive increase (Fig. 4B), while Bcl-2 showed a negative relation against Bax after serum-free treatment (Fig. 4D). More importantly, myricetin stimulated the expression of Bax, while suppressing the expression of Bcl-2. The ratio of Bax against Bcl-2 showed an increasing tendency as same as data from Fig. 4A and B (data not shown).

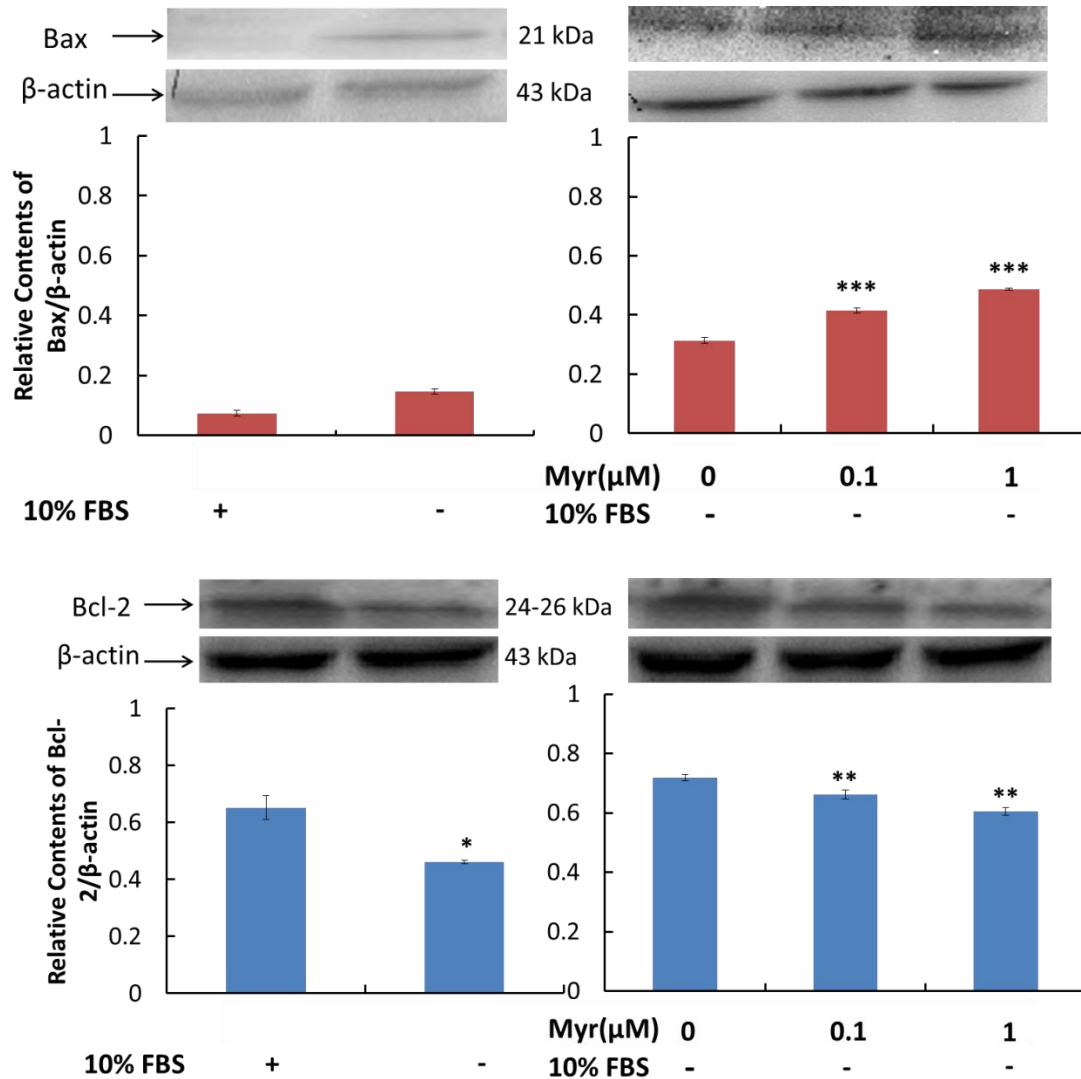


Fig. 4. Pro-apoptotic factor Bax and antiapoptotic factor Bcl-2 was determined by western blot. Both contents in PC12 cells treated with 0 and 10% FBS and with 0, 0.1

and 1 μ M myricetin under serum deprivation condition for 72 h. Error bars indicate SEM ($n \geq 3$). ** and *** indicate significant differences at $p < .01$ and $p < .001$.

2.4.5. Activation of tumor suppressor p53 gene

Fridman and Lowe (2003) have elucidated that tumor suppressor p53 gene promoted apoptosis through transcription-dependent and independent mechanisms. Herein, myricetin enhanced the expression of p53, confirming the promotion of the apoptosis induced by serum deprivation following a dose-dependent manner (Fig. 5B). Interestingly, Chipuk et al. (2004) reported that p53 gene can also directly regulate pro-apoptotic Bax activation so as to mediate mitochondrial membrane permeabilization and apoptosis.

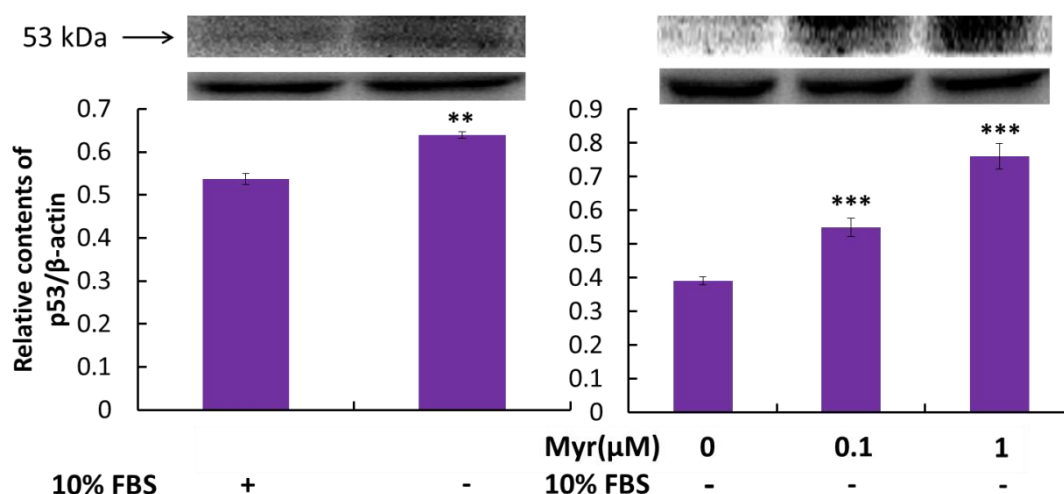


Fig. 5. Increasing expression levels of p53 in PC12 cells treated with 0 and 10% FBS (A) and with 0, 0.1 and 1 μ M/mL myricetin under serum deprivation condition (B) for 72 h were determined by western blot. Error bars indicate SEM ($n \geq 3$). ** and *** indicate significant differences at $p < .01$ and $p < .001$, respectively.

2.4.6. Caspase cascade reaction of caspase-3 and -9

Many researchers have already proved that p53 triggers apoptosis by induction of caspase cascade reaction through mitochondrial cytochrome c release (Schuler et al., 2000). We found that caspases 3 and 9 were activated after serum-free medium

culture. Subsequently, myricetin enhanced caspases 3 (Fig. 6A and B) and (Fig. 6C and D) expression as the dose increases.

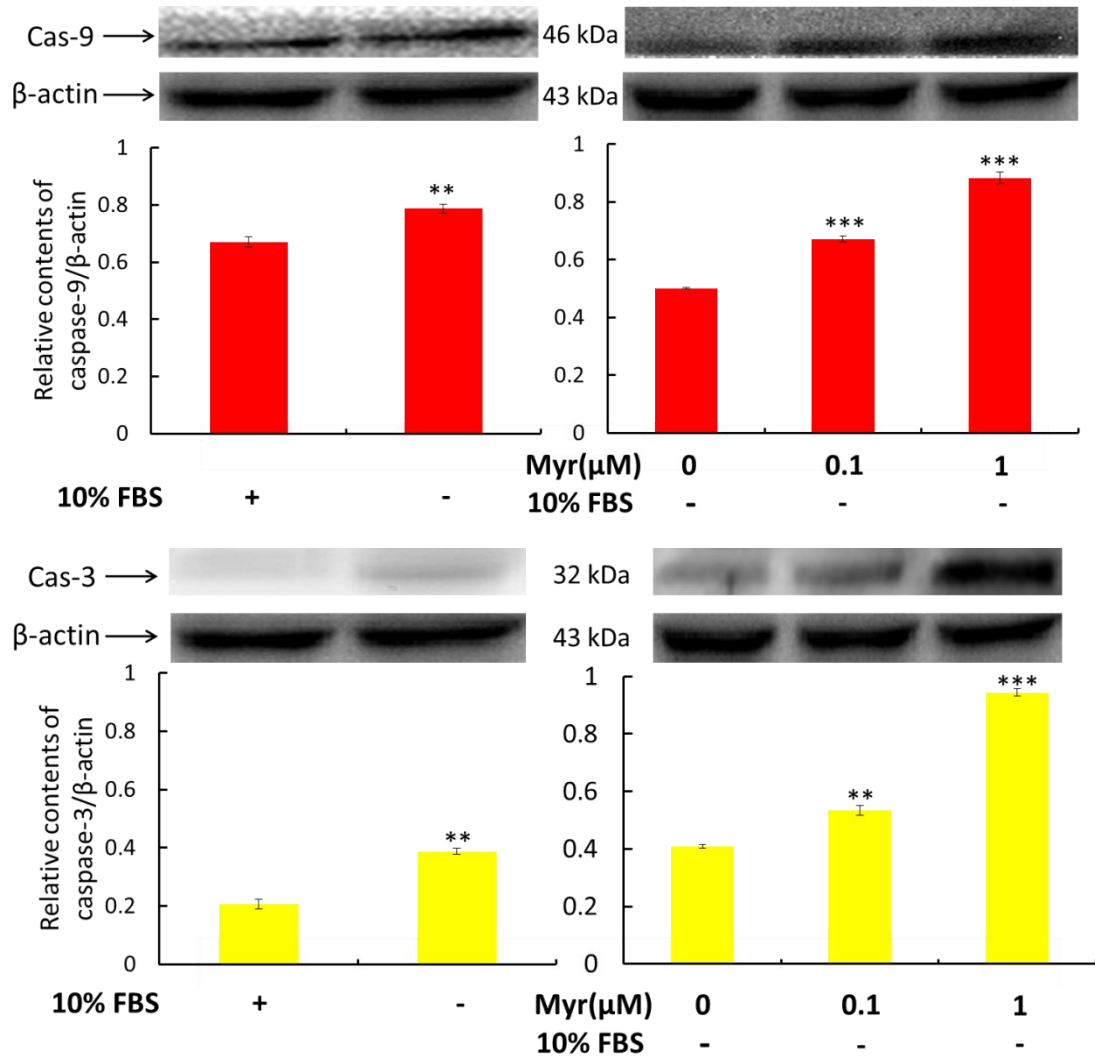


Fig. 6. Cleaved caspase 3 and caspase 9 was determined by western blot. Both contents in PC12 cells treated with 0 and 10% FBS (A and C) and with 0, 0.1 and 1 μ M/mL myricetin under serum deprivation condition (B and D) for 72 h. Error bars indicate SEM ($n \geq 3$). ** and *** indicate significant differences at $p < .05$, $p < .01$ and $p < .001$.

2.5. Discussion

In this study, myricetin showed its enhancive effect on the apoptosis induced by serum deprivation in PC12 cells. A synergistic effect was reported by Xu et al. (2013) that myricetin enhanced the apoptosis of prostate cancer cells (PC-3 cells) in a dose dependent manner in combination with myricetrin. Wang et al. (2014) also reported that the combination of myricetin with 5-fluorouracil chemotherapy could enhance tumor chemosensitivity of esophageal cancer EC9706 cells, and it also implied myricetin could be a potential chemosensitizer for esophageal cancer therapy. Recently, it was reported that the combination of myricetin and methyl eugenol with cisplatin is a potential clinical chemotherapeutic approach in human cervical cancer (Yi et al., 2015). Moreover, myricetin could induce cytotoxicity and DNA condensation in human colon cancer cells (HCT-15 cells) in a dose-dependent manner. It increased the Bcl-2-associated X protein/B-cell lymphoma 2 ratio, but not cleavage of caspase-3 and caspase-9, and induced the release of apoptosis-inducing factor from mitochondria simultaneously (Kim et al., 2014).

While the enhancements of apoptosis using myricetin under different conditions were already reported, a study on whether myricetin can enhance apoptosis induced by serum deprivation is not yet explored. Herein, serum starvation induced apoptosis did happen due to the lack of neurotrophin. After 72 h, increased significant cytotoxicity was observed from the myricetin-treated PC12 cells (Fig. 1). In order to prove the enhancement on the serum deprived induced apoptosis, the expression of several related proteins such as p53, Bax, Bcl-2, cytochrome c, caspase-9 and -3 was determined by western blot assay. As a result, the enhancement of myricetin on apoptosis induced by serum deprivation in PC12 cells was determined to be following the mitochondrial signaling pathway. Herein, expression level of cytosolic cytochrome c is higher in serum free group than control group, while much higher in the myricetin-treatment groups with doses increasing (Fig. 3). The increasing level of Bax was due to the increasing level of p53 (Fig. 5), while anti-apoptotic Bcl-2 protein exhibited its negative correlation to Bax as shown

in Fig. 4. Fridman and Lowe (2003) pointed out the loss of p53 attenuated the protein expression of downstream targets and the activation of p53 can be induced by DNA damage and repair, cell cycle arrest, hypoxia, cell senescence and apoptosis. The regulation of activated p53 in response of DNA damage is related with the increasing level of activated p53 to bind DNA and mediate transcriptional activation by triggering phosphorylation, de-phosphorylation and acetylation on the p53 polypeptide (Lakin and Jackson, 1999). Meanwhile, it also has been reported that tumor suppressor gene p53 cannot only regulate cell cycle progression, but also trigger apoptosis after its being activated (Meikrantz and Schlegel, 1995). In fact, p53 which functions on cell cycle regulation and DNA repair depends on the transactivation of its response genes like p21 (el-Deiry, 1998), but the tumor suppressing activity of p53 does not rely on its transactivation function (Crook et al., 1994). It is more crucial to point out that p53 triggered apoptosis in the way of regulating transcription of pro-apoptotic Bcl-2 family members like Bax (Miyashita and Reed, 1995). Moreover, Schuler et al. (2000) have illustrated that p53 induces apoptosis by death-acceptor independent pathway. Also, the release of mitochondrial cytochrome c was in response to the transcriptional activation of pro-apoptotic Bax protein. In this study, it actually increased the ratio of Bax versus Bcl-2 dimers to promote the downstream genes expression in the caspase cascade reaction and apoptogenic protein release. The significant difference of related protein expression was showed between control group and FBS(-) treatment, and more enhancement was found with the dose increase of myricetin from western blot results of p53, Bax, Bcl-2 and cytochrome c. Simultaneously, the increasing level of caspase 9 accelerated the activation of caspase 3 in Fig. 6. The DNA damage level was shown by DNA fragmentation with doses of myricetin increasing possibly because of the disruption on DNA transcription activation due to the activation of p53. Precisely, p53 activation evokes a series of complicated and closed link to execute apoptosis by the release of cytochrome c as a result of the transcriptional activation of Bcl-2 family members, especially pro-apoptotic Bax gene and anti-apoptotic Bcl-2 gene. The released cytosolic cytochrome c which binds with the

adaptor molecular Apaf-1, forms a complex with oligomerized caspase 9 in the presence of ATP to activate caspase 3 and sequentially cleave cellular death substrates. Herein, DNA fragmentation and the release of cytochrome c characterized the occurrence of apoptosis. The following mechanism was clarified in figure 7. Moreover, myricetin exhibited enhancement on the apoptosis induced by serum deprivation in PC12 cells through related protein expression.

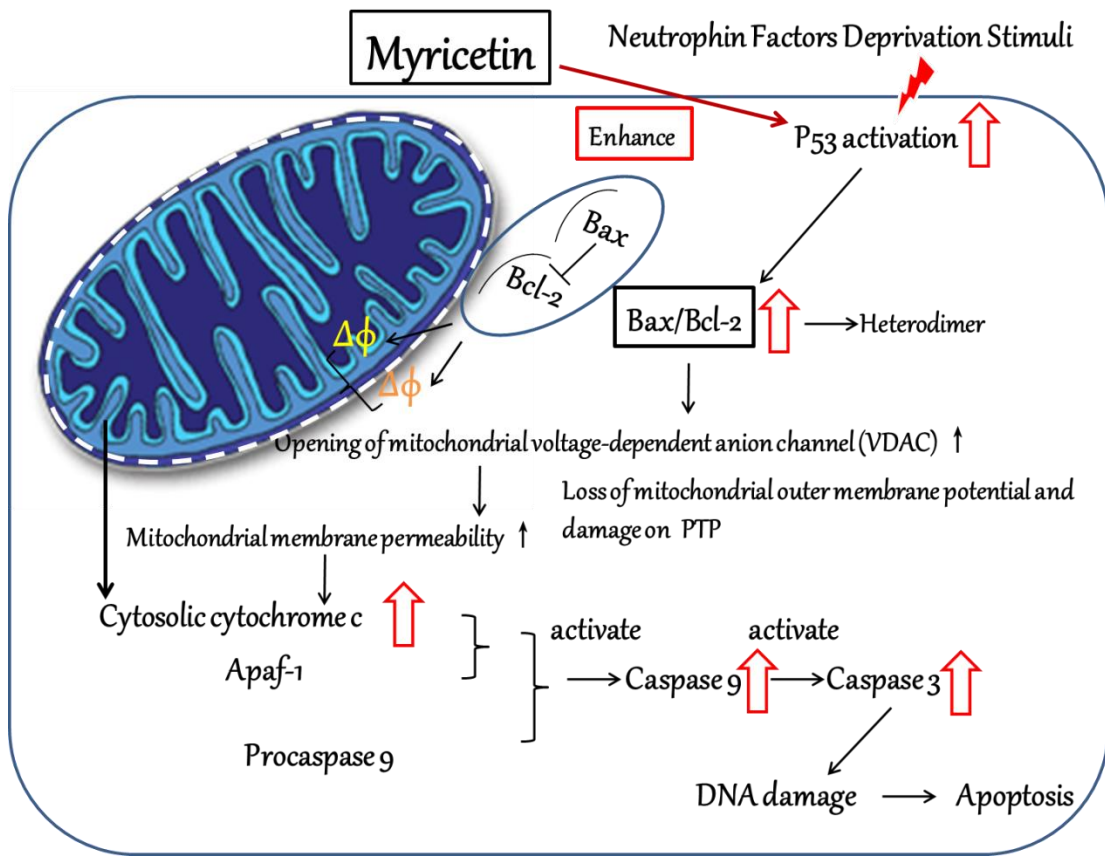


Fig. 7 The mechanism of apoptosis induced by serum deprivation mediated by intrinsic or mitochondrial signaling pathway in PC12 cells was showed. Myricetin exhibited its enhancement effect on the apoptosis.

2.6. Conclusion

This study provides new evidence to support the promotion of the anti-tumor effect of myricetin under serum deprivation in undifferentiated PC12 cells via dose-dependent manner. Additionally, it is necessary to point out that myricetin may have an influence on undifferentiated and differentiated cell through different mechanisms. Therefore, further study will be essential to distinguish cell types so as to focus on understanding corresponding mechanism in detail.

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Chapter 3

Myricetin promotes apoptosis mediated by p53 activation in breast cancer MCF-7 cell lines

3.1 Abstract

Polyphenols are abundant in our daily food. Myricetin, as a common flavonoid compound, widely exists in many fruits, vegetables or tea. Our previous study reported the enhancement effect on apoptosis induced by serum deprivation in PC 12 cells mediated by mitochondrial signaling pathway. Although there are already researches about myricetin, few study investigated the effect of myricetin on breast cancer, MCF 7 cells, specifically its anti-cancer mechanism. In the present study, dose-dependent inhibition in the growth of MCF-7 cells by myricetin was evaluated using MTT assay and trypan blue staining assay. Thereafter, decreasing GSH levels were determined indicating higher ROS generation as the dosage of myricetin is increased. Moreover, the expression of apoptotic related proteins was determined via western blotting analysis. The activation of tumor suppressor protein p53 showed an increasing dose-dependently. This had a negative relation to the activation of NF- κ B. Simultaneously, it induced the transcriptional activation of Bax versus Bcl-2 dimer. It was found that the adaptor receptor Apaf-1 and caspase-3 was also activated when apoptosis was induced by myricetin treatment. It could directly reflect the release of cytochrome c because Apaf-1 binds with released cytosol cytochrome c, forms a complex with oligomerized caspase 9 in the presence of ATP to activate caspase 3 and sequentially cleave cellular death substrates. In conclusion, myricetin exhibited its potent anti-cancer effect on breast cancer MCF 7 cells through p53 mediated apoptotic signaling pathway. These results can be used in researches regarding cancer therapies, which target the decrease of side effects from cancer drugs.

3.2. Introduction

According to a data recently reported in the Medical News Today by Christian Nordqvist, breast cancer is the most common invasive cancer in women, and the second main cause which induced cancer death in women, after lung cancer (1). Until now, the severity of breast cancer is still not adequately paid attention. Patients who have suffered breast cancer commonly have typical symptoms including a lump or thickening of the breast, and changes to the skin or the nipple (1). The risk factors are primarily pointed to dietary structures, living standards and lifestyle factors such as alcohol intake and smoking (2). Recently, new data is reported that the morbidity of breast cancer is increasing year by year, and moreover the age onset shows a much younger trend (3). The treatments that are available include surgery, radiation therapy, chemotherapy, hormone therapy and targeted drug therapy. However, so many unexpected severe side effects such as fatigue, darkening of the breast skin, irritation of the breast skin, nausea, vomiting, loss of appetite, fatigue, sore mouth, hair loss, and a slightly higher susceptibility to infections have been reported (4). Thus, there is an alarming need to find a relatively safer with minimal to none side effect compound and develop mechanism based methods for cancer treatment or protection.

Myricetin, which is a type of polyphenol that can be taken daily, is abundant in vegetables, fruits, tea, wine and other beverages (5). The median daily intake in western diet is up to 10 mg (6). Previous studies have showed that myricetin possesses various properties including anti-oxidant, pro-oxidant, DNA degradation, anti-carcinogen, mutagen, anti-viral, inhibition of DNA/RNA replication and repair, anti-thrombotic, anti-atherosclerotic, anti-diabetic and other therapeutic effects. Anti-cancer effect is regarded as a relatively important property, which has been reported by most studies. Recently, Cao J et al., (2017) (7) reported the anti-cancer property of myricetin by inhibiting the phosphorylation of mTOR in HepG2 cells to induce protective autophagy. Another study also demonstrated anti-cancer activity of myricetin against human papillary thyroid cancer cells involves mitochondrial

dysfunction-mediated apoptosis. It was found that myricetin induced cytotoxicity and DNA condensation in SNU-790 HPTC cells in a dose-dependent manner. Simultaneously, myricetin induced the apoptosis in SNU-790 HPTC cells by a series of processes including up-regulation of caspase cascades activation, Bax versus Bcl-2 expression ratio, the release of apoptosis-inducing factor (AIF) and altered the mitochondrial membrane potential (8). Additionally, Yang C et al., (2017) (9) also investigated that myricetin suppresses invasion and promotes cell death in human placental choriocarcinoma cells by inducing oxidative stress. Myricetin induced a series of changes including ROS production, lipid peroxidation, glutathione depletion, and loss of mitochondrial membrane potentials. And invasive and pro-angiogenic properties of malignant JAR and JEG-3 trophoblast cells were attenuated by myricetin treatment via MAPK and PI3K/AKT signaling pathways. Moreover, myricetin exhibited synergistic anti-proliferative effects with some chemotherapeutics, etoposide and cisplatin on choriocarcinoma cells. From previous studies, it can be said that myricetin possesses its anticancer effect through the generation of reactive oxygen species (ROS) and subsequently destroys cellular redox balance and induces the apoptosis in cancer cells (10). Generally, myricetin tends to exhibit anti-carcinogenic effect by inducing cell death to protect cancer cells from proliferation mediated by some related signaling pathways.

Cross talking between proteins is a complicated and important process to complete a series of apoptotic response. P53, functions as the extremely important tumor suppressor, acts in response to diverse forms of cellular stress to mediate a variety of anti-proliferation processes (11). The activation of p53 can be induced by DNA damage, hypoxia, or aberrant oncogene expression to promote cellular senescence and apoptosis (11). Therefore, the cellular immortalization, genomic instability, and abnormal survival are stimulated by the activation of p53, as a result, it affects proliferation and evolution of damaged cells.

NF- κ B, a protein complex which can control DNA transcription, has showed its potential on anti-apoptosis and pro-apoptosis. Previously, it was reported that p53

could induce activation of NF- κ B, and loss of NF- κ B activity so as to suppress p53-mediated apoptotic response (12). However, NF- κ B exhibits anti-apoptotic activity in many occasions such as TNF α -mediated signaling pathways (13). Gill A. Webster et al (1999) have already reported suppression of p53 could induce the activation of NF- κ B in tumorigenesis and other diseases.

Caspases are a series of important mediators of programmed cell death (apoptosis) (14). Particularly, caspase 3, can be activated in a protease cascade which leads to inappropriate activation or rapid disablement of key structural proteins and crucial signaling, homeostatic and repair enzymes (15). In addition, caspase 3 is a frequently activated death protease, catalyzed the specific cleavage of many key cellular proteins. In our recent study (16), it was showed that serum starvation induced the apoptosis in PC12 cells while, myricetin treatment enhanced the apoptosis. The activation of p53 induced a series of changes in the molecular biological mechanism of apoptosis including cytochrome c release, expression of Bax, Bcl-2, caspase 3 and 9. In fact, serum deprivation, as external stimuli, activated the activation of p53, which subsequently increased the ratio of Bax versus Bcl-2 dimers to promote the downstream genes expression in the caspase cascade reaction and apoptogenic protein release. Precisely, p53 activation evokes a series of complicated and closed link to execute apoptosis by the release of cytochrome c as a result of the transcriptional activation of Bcl-2 family members, especially pro-apoptotic Bax gene and anti-apoptotic Bcl-2 gene. The released cytosolic cytochrome c which binds with the adaptor molecular Apaf-1, forms a complex with oligomerized caspase 9 in the presence of ATP to activate caspase 3 and sequentially cleave cellular death substrates.

Previous study already reported that, myricetin showed its anti-cancer effect on human breast cancer MCF-7 cells through inhibition of p21-activated kinase 1 and subsequent regulation of downstream signaling of the β -catenin pathway. However, the proof of the reason why myricetin possess anti-cancer effect is still not sufficient. As such, the goal of this chapter is to further study the effects of myricetin in MCF-7 cells, through other related pathways.

3.3. Materials and methods

3.3.1. Chemicals

RPMT-1640 and fetal bovine serum (FBS) was purchased from Wako and HyClone (Rockville, MD, USA). Biotinylated goat anti-mouse IgG whole antibody and ECL western blotting detection reagent were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). Biotinylated donkey anti-rabbit immunoglobulin was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Anti-caspases 3 was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Anti-p53 was bought from Cell Signaling Technology (Beverly, MA, USA). Trypan blue solution (0.4%) was purchased from Bio-Rad (Hercules, CA, USA). Myricetin, was purchased from Tokyo Chemical Industry Company (Tokyo, Japan). All other chemicals were of analytical grade.

3.3.2. Cell culture

Human MCF 7 breast cancer cells (ECACC 86012803, Culture Collections, Public Health England, local distributor: DS Pharma Biomedical, Cat. No. EC86012803) were maintained in 5 mL RPMI-1640 and 10% fetal bovine serum. Trypsin-EDTA solution was bought from Sigma. MCF 7 cells were cultured in a humidified incubator at 37 °C and 5% CO₂ with 10% of FBS 24 h prior to treatment. Myricetin was diluted with ethanol into two concentration levels of 10, 20 and 40 mM. Thereafter, four groups were set including control, 10 µM (5 mL of 10% fetal bovine serum medium with 5 µL of 10 mM myricetin), 20 µM (5 mL of 10% fetal bovine serum medium with 5 µL of 20 mM myricetin) and 40 µM (5 mL of 10% fetal bovine serum medium with 5 µL of 40 mM myricetin). These administration amounts were decided on the basis of daily intake amounts of myricetin explained by Jayakumar et al. (2014), and Thilakarathna and Rupasinghe (2013). The treatment period lasted for 48 h.

3.3.3. MTT assay

One Iwaki 25 cm² flask was used as the original subculture for MTT assay. The adherent MCF 7 cells were harvested by trypsin-EDTA solution followed by 5 min incubation at 37°C with 5% CO₂ and then centrifuged at the speed of 1500 rpm for 3 min. Thereafter, the supernatant was discarded. The cells were resuspended with 1 mL RPMI plus FBS medium and seeded into 96-well plates at a density of 1×10^4 cells (90 µL of total volume per well). After 24 h incubation, the cells were treated with 10 µL sample solutions (1 µL of myricetin and 9 µL of RPMI plus FBS medium). Then the cells were incubated at 37°C with 5% CO₂ incubator for 24, 48 and 72 h. After corresponding incubation time, MTT was aspirated, and the formazan crystals were dissolved in 100 µL DMSO. The optical density at 570 nm was measured using a Thermo Labsystems Multiskan JX plate reader. IC₅₀ values were calculated by Graphpad Prism7.

3.3.4. GSH (Glutathione) level

Glutathione levels were performed by Rahman et al. (2017) and Kihara et al. (2012). MCF 7 cells were treated with 10 µM, 20 µM and 40 µM of myricetin for 48 h. After 48 h, the cells were harvested by trypsin-EDTA, and washed with 1× phosphate-buffered saline (PBS). Thereafter, the cells were lysed with 120 µL lysis buffer, and kept on ice at room temperature for 10 min. Two freeze-thaw sonication cycles were utilized for cell membrane disruption. Then the disrupted cells were centrifuged at speed of 700 × g for 10 min for collecting the supernatant. Protein assay dye reagent (Bio-Rad, Hercules, CA, USA) was used for spectrophotometrically measuring the total protein content. 2.5 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, pH 7) assay kit was used for determining GSH levels. The absorbance was measured at 405 nm wave length by using Microplate Reader after adding DTNB to the cell lysate solution. The GSH levels were calculated followed by 0.015 absorbance equivalent to 1 µM of GSH. The experiments were performed in triplicate.

3.3.5. Western blot analyses of apoptotic gene expression

For determination of β -actin, Bax, Bcl-2, p53, caspases-3, NF- κ B, lysis buffer (100 mmol/L HEPES, 1 mol/L NaCl, 300 mmol/L EGTA, 0.1 M PMSF, 100 mmol/L Na₃VO₄, 10 mmol/L Na₂MgO₄, 100 mmol/L 2-glycerophosphoric acid, 1 mmol/L MgCl₂, 100 mmol/L DTT, 100 mmol/L NaF, and triton X-100) was used to collect the protein fraction after two cycles of sonication. Thereafter, the approximately equal content of protein was applied and separated on 12.5% polyacrylamide gel, and the electrophoresed proteins were transferred to nitrocellulose membranes with a semidry blotting system, type AE6678 (ATTO, Tokyo, Japan). The membranes were incubated overnight at 4 °C in 5% skim milk as a blocking agent. The membranes were incubated for 1 h at 37 °C with the primary antibodies, washed three times (5 min per time) and then incubated with the secondary antibody for 1 h in the 37°C incubator. After antibody reaction, related protein expression was imaged in the way of protein bands with an enhanced chemiluminescence imaging system (ChemiDoc XRS, Bio-Rad). The band density level was measured with Image J software.

3.3.6. Statistical analysis

All the experiments were performed at least three times. The data were analyzed for significant difference using Student's t-test and one-way ANOVA. P values $\leq .05$ were considered to indicate statistically significant differences. The mean $\pm$ SD values were calculated for all variables.

3.4. Results

3.4.1. Myricetin inhibited the viability of MCF-7 cells

To evaluate the effect of myricetin on MCF-7 cell which was exposed to 0.5, 5, 10, 20, 25, 40, 50, 100, 200 μM for 24, 48 and 72 h, MTT assay was performed to analyze the inhibitory effect on MCF-7 cells following dose and time point. Herein, myricetin exhibited its inhibitory effect on MCF 7 cells in a dose and time dependent manner (fig.8). The inhibitory effect is higher in the 48 h group than 72 h group below 20 μM . However, the opposite effect was showed above 20 μM . The IC_{50} was estimated to be 44.0, 29.5 and 21.3 μM at 24, 48 and 72 h, respectively.

	24 h	48 h	72 h
IC_{50} (μM)	44.0	29.5	21.3

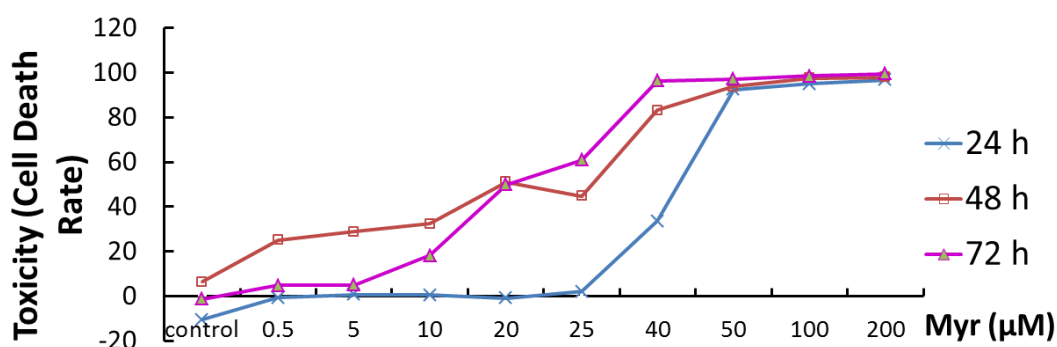


Figure 8. MCF-7 cells were treated with the indicated concentrations of myricetin for 24 h (x), 48 h ($\square$) and 72 h ($\triangle$), and the inhibitory effect was determined by the MTT assay. Data represent the means $\pm$ SD of two independent experiments performed eight times.

3.4.2. Cell viability

Cell viability was determined by trypan blue staining assay. MCF-7 cells were treated with 10, 20, 40 μM of myricetin for 48 h. Significant changes were found after trypan blue staining assay determination(fig.9).

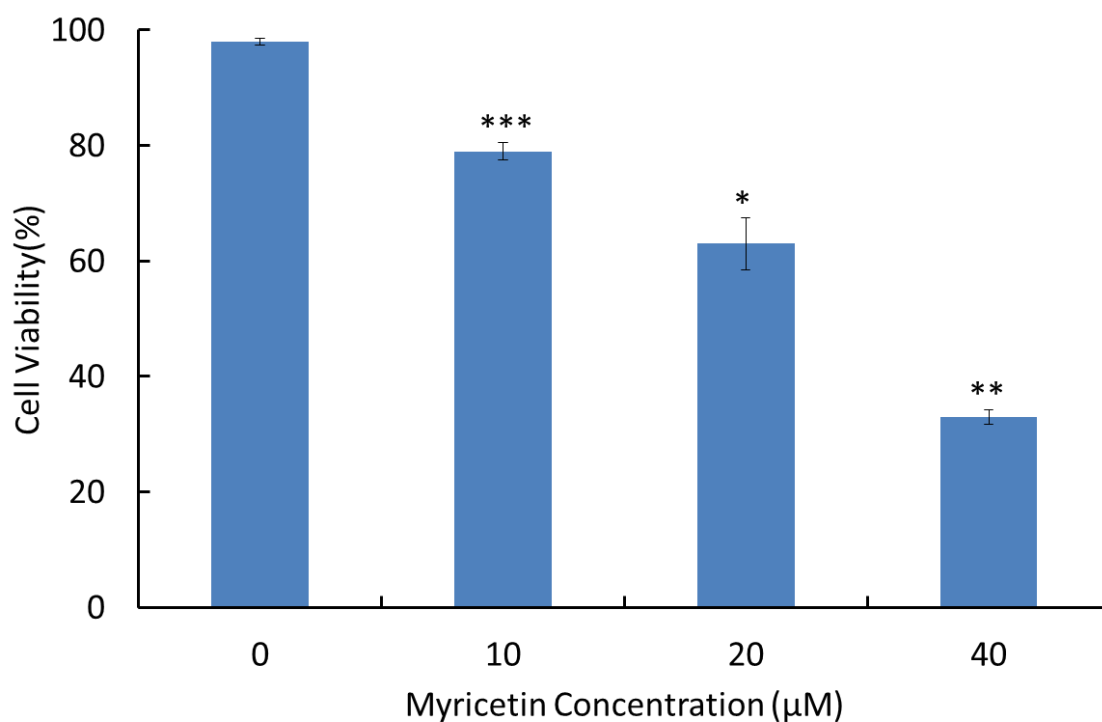


Figure 9. MCF-7 cells were treated with various doses of myricetin (10, 20, 40 μM). The cell viability was determined by typan blue staining assay after 48 h treatment. Error bars indicate SEM ($n \geq 3$). *, ** and *** indicate significant differences at $p < 0.05$, 0.01 and 0.001, respectively.

3.4.3. GSH level

From the results of MTT assay and cell viability, it is known that myricetin showed toxicity on MCF-7 cells in a dose and time dependent manner. This toxicity disrupted the balance of redox in the intracellular environment. Herein, a decreasing trend of GSH levels implied more glutathione was oxidized to GSSG (fig.10) and indirectly reflected that the ROS generation was stimulated with increasing dose.

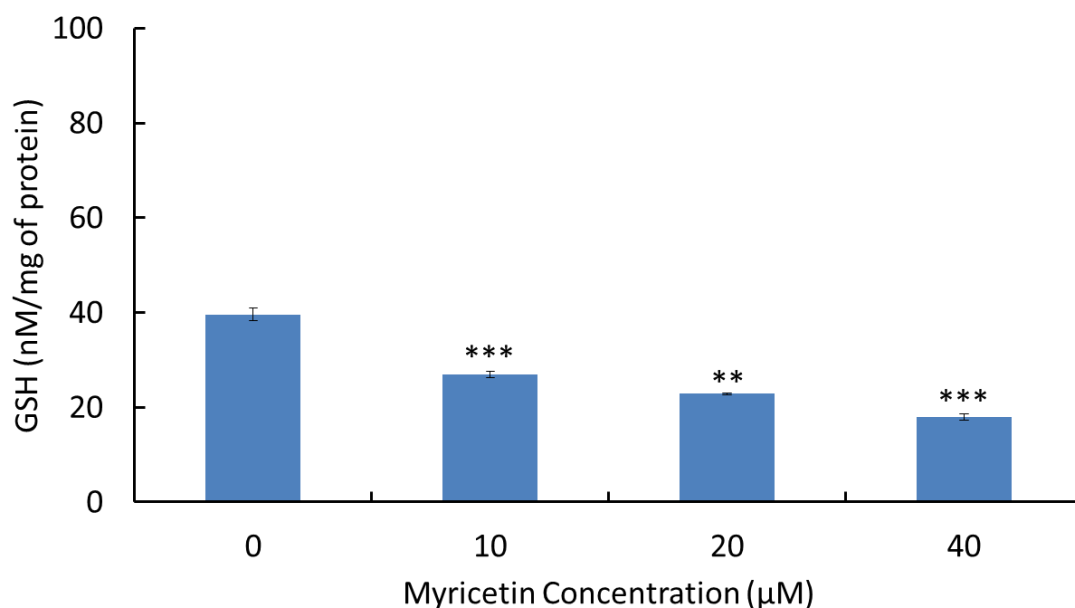
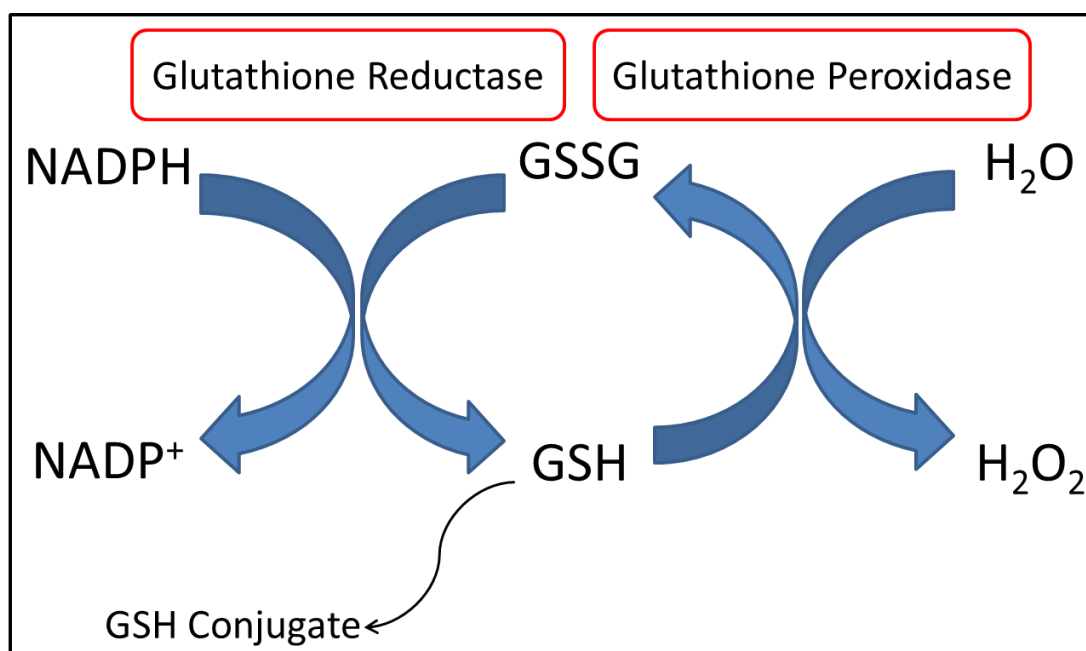


Figure 10. MCF-7 cells were treated with various doses of myricetin. The GSH levels were determined by Microplate Reader after 48 h. Error bars indicate SEM ($n \geq 3$). ** and *** indicate significant differences at $p < .01$ and 0.001.



3.4.4. Activation of tumor suppressor p53 gene

Previous study reported that the tumor suppressor p53 inhibits cell growth through activation of cell-cycle arrest and apoptosis. In the present study, the expression of p53 exhibited an increasing trend after treatment of increasing doses

of myricetin (fig.11). This result suggest the occurrence of apoptosis.

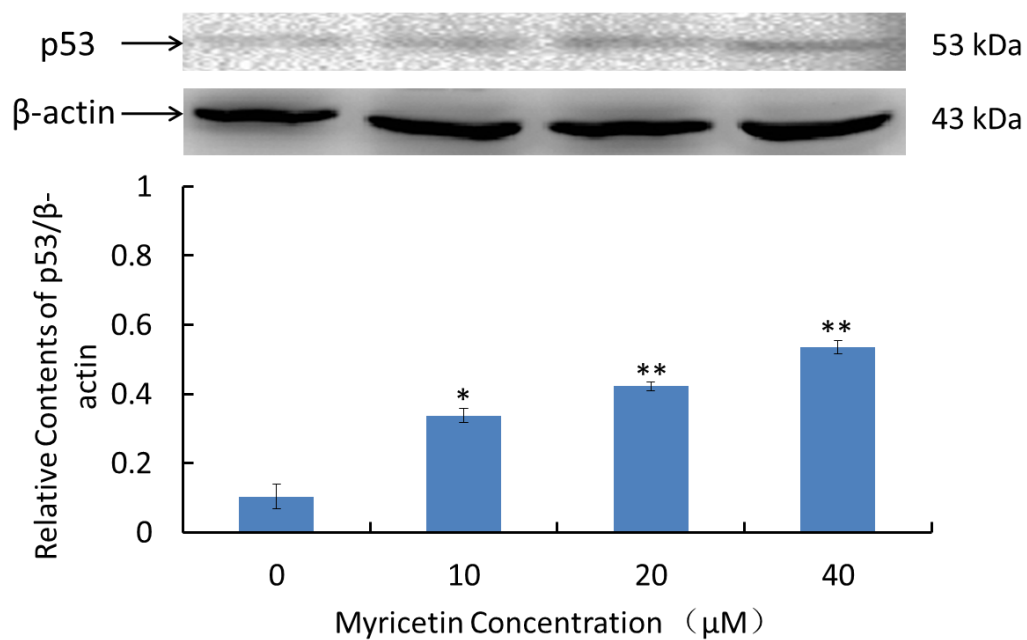


Figure 11. The expression of p53 in MCF-7 cells treated with 10, 20 and 40 μM myricetin for 48 h was determined by western blot. Error bars indicate SEM ($n \geq 3$). * and ** indicate significant differences at $p < 0.05$ and 0.01.

3.4.5. Activation of NF-kappa B

NF- κ B, plays a critical role in managing the immune response to infection. Herein, the expression of NF- κ B showed a decreasing trend (fig.12). It has been already reported NF- κ B has an anti-apoptotic potential in many systems. From fig.12, an attenuated trend was clearly found in a dose dependent manner.

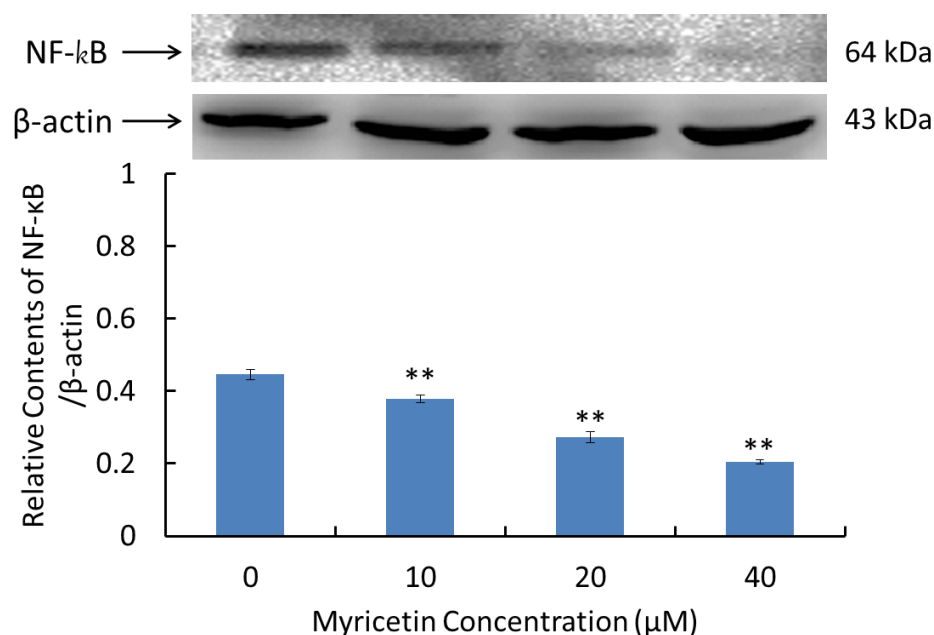


Figure 12. The expression of NF- κ B in MCF-7 cells treated with 10, 20 and 40 μ M myricetin for 48 h was determined by western blot. Error bars indicate SEM ($n \geq 3$). ** indicates significant differences at $p < 0.01$.

3.4.6. Expression of pro-apoptotic gene Bax, anti-apoptotic gene Bcl-2 and Bcl-x

It was reported in our past study that p53 induced the transcriptional activation of Bax versus Bcl-2 dimers, and then evoked a series of changes including cytochrome c release and downstream caspase cascade signaling. In the present study, the opposite trend of expression level was observed between Bax and Bcl-2 as shown in fig.13. A decreasing trend of Bcl-x expression was observed in a dose dependent manner (fig.14).

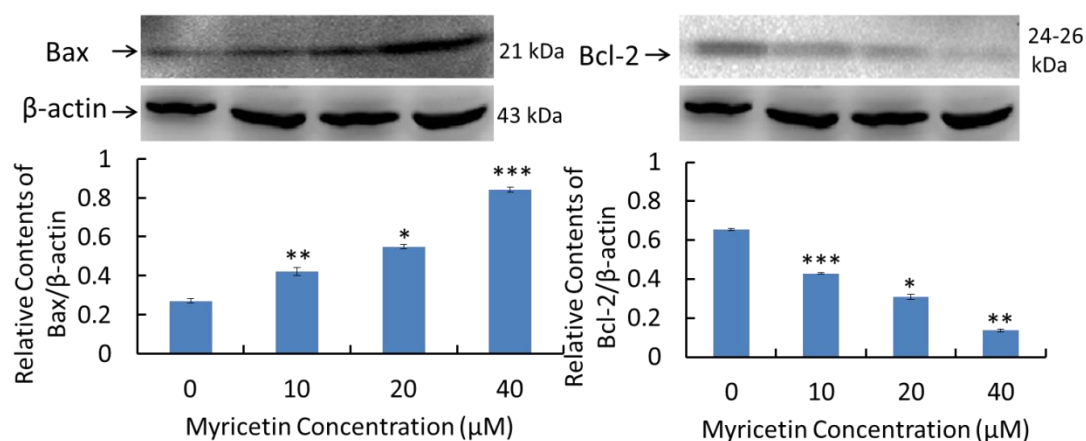


Figure 13. The expression of pro-apoptotic factor Bax and antiapoptotic factor Bcl-2 was determined by western blot. Both contents in MCF-7 cells treated with 10, 20 and 40 μM myricetin for 48 h. Error bars indicate SEM ($n \geq 3$). *, ** and *** indicate significant differences at $p < 0.05$, 0.01 and < 0.001 , respectively.

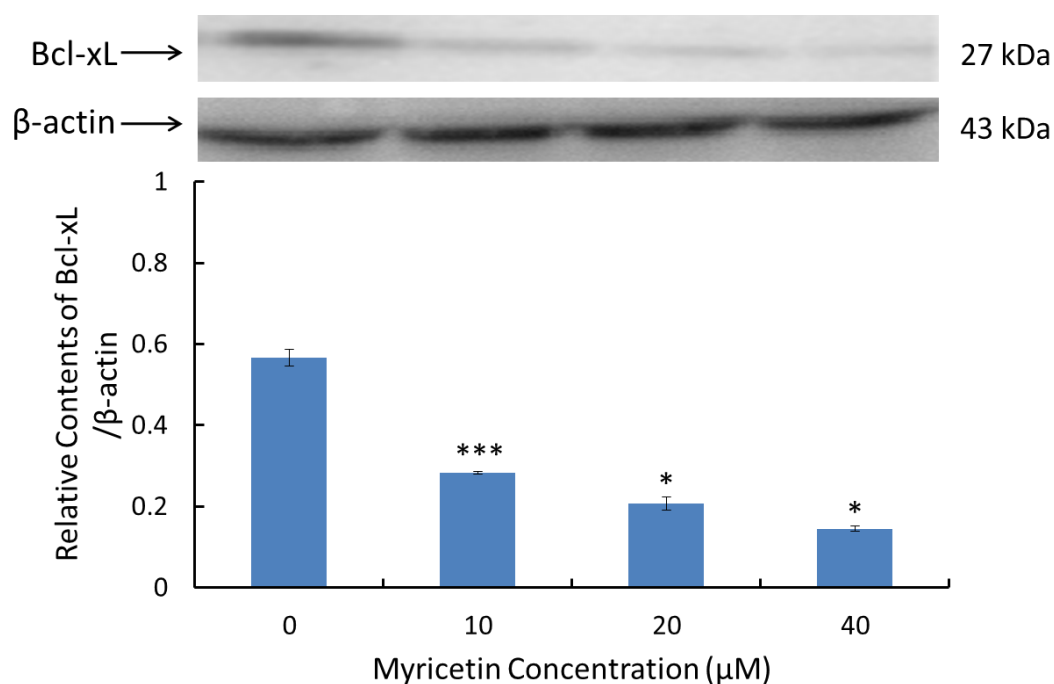


Figure 14. The expression of antiapoptotic factor Bcl-xL was determined by western blot. Both contents in MCF-7 cells treated with 10, 20 and 40 μM myricetin for 48 h. Error bars indicate SEM ($n \geq 3$). * and *** indicate significant differences at $p < 0.05$ and $p < 0.001$.

3.4.7. The expression of Apaf-1

Previous study has already reported that the adaptor molecular Apaf-1 binds

with the released cytosolic cytochrome c and, forms a complex with oligomerized caspase 9 in the presence of ATP to activate caspase 3 and sequentially cleave cellular death substrates. Herein, the activation level of Apaf-1 was determined by western blot to indirectly access the expression level of cytochrome c. An increasing trend was observed after treatment with increasing doses as shown in fig. 15.

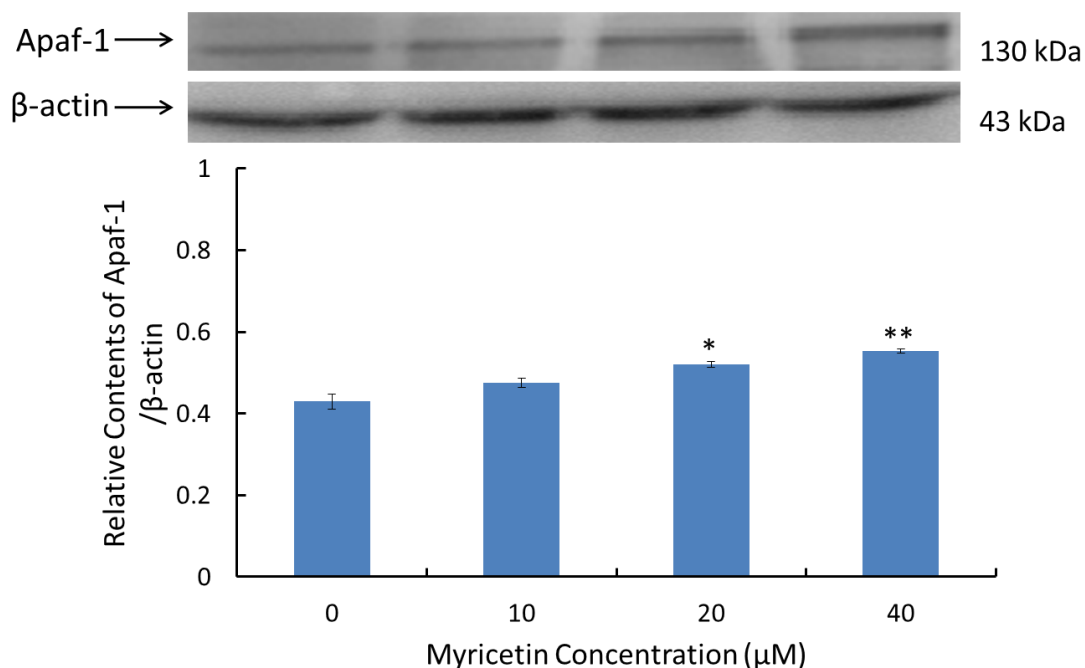


Figure 15. The activation of Apaf-1 was determined by western blot. Both contents in MCF-7 cells treated with 10, 20 and 40 μM myricetin for 48 h. Error bars indicate SEM ($n \geq 3$). * and ** indicate significant differences at $p < 0.05$ and 0.01 .

3.4.8. Caspase cascade reaction of caspase-3

Caspase-3, an important caspase protein in caspase cascade reaction, interacts with caspase-8 and caspase-9 to reflect apoptosis occurrence. From fig.16, caspase-3 was activated after treatment with 10 μM myricetin. Moreover, an increasing trend was observed in a dose dependent manner.

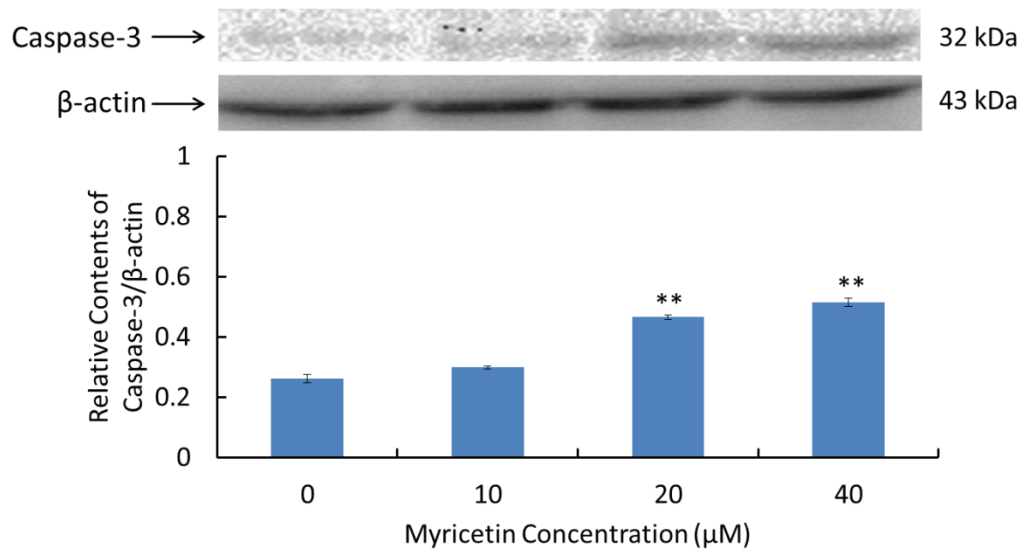


Figure 16. Caspase-3 was determined by western blot. Both contents in MCF-7 cells treated with 10, 20 and 40 μM myricetin for 48 h. Error bars indicate SEM ($n \geq 3$). ** indicates significant differences at $p < 0.01$.

3.5. Discussion

Results from the cell viability determination via MTT assay and typan blue staining assay implicates the induction of cell death after myricetin treatment in MCF-7 cells. It is then confirmed that myricetin induced apoptosis, not necrosis, after protein expression determination of NF- κ B, p53, Bax, Bcl-2, Apaf-1 and caspase-3 through western blot analysis. Moreover, the redox balance disruption in the cellular microenvironment possibly because of reactive oxygen species (ROS) generation was directly determined via measurement of GSH levels. Subsequently, this activates p53 and NF- κ B which exhibits a negative relation to p53. The activation of p53 induced transcriptional activation of Bax versus Bcl-2 dimer. The expression of Bax and Bcl-2 increased opening of mitochondrial voltage-dependent anion channel (VDAC) and induced the loss of mitochondrial outer membrane potential and damage on PTP. As a result, mitochondrial membrane permeability was disrupted. Mitochondrial cytochrome c was released into cytosol. Cytosolic cytochrome c, binds with Apaf-1, forms a complex with procaspase-9 to activate caspase-3. These results suggest that myricetin-induced apoptosis in MCF-7 cell lines accompanied a series of molecular biological changes including glutathione peroxidase to GSSG and expression of apoptosis-related proteins. Previous studies reported many other toxic effect of polyphenol on different cell lines. It was reported in the Applications in Functional Foods that diosmetin and luteolin showed toxicity on human colon cancer colon205 cell line with the IC₅₀ values of 82.9 and 96.9 μ M, respectively. Additionally, Katrin et al., (2018) reported the toxicity of fisetin and quercetin on human adeocarcinoma A549 cell line with the IC₅₀ values of 127.9 and 72.2 μ M, respectively. Herein, the IC₅₀ values of myricetin on breast cancer MCF-7 cell line was 44, 29.5 and 21.3 μ M for 24, 48 and 72 h, respectively. Myricetin showed relatively higher levels of toxicity than some polyphenols. It is reported that polyphenols can induce apoptosis by intrinsic pathway, and the anticancer mechanism of most anticancer drug or polyphenols depends on intrinsic signaling pathway (17). In the present study, like other polyphenols, myricetin, was observed to induce intrinsic signaling pathway, critical

role during apoptosis. Results showed that myricetin induced apoptosis mediated by p53 activation in breast cancer MCF-7 cell lines.

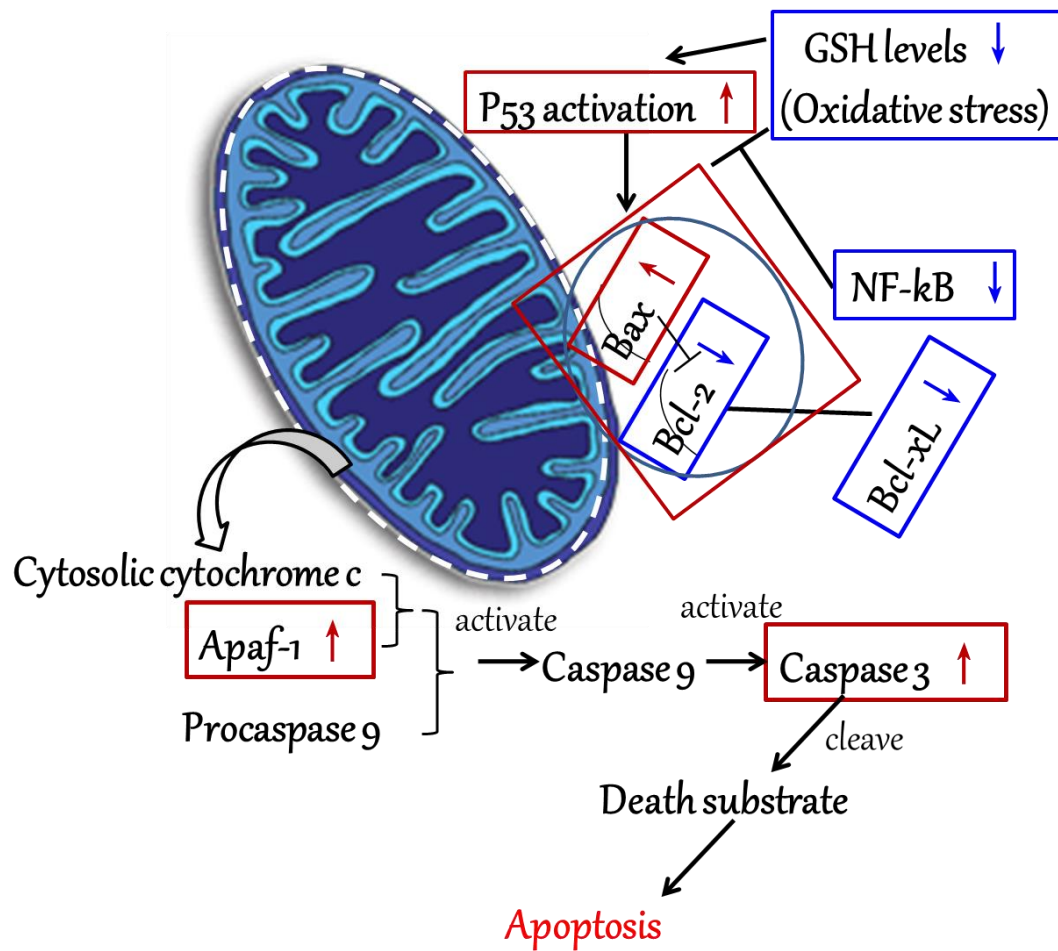


Fig. 17 The mechanism of apoptosis which induced by myricetin in MCF-7 cell lines mediated by intrinsic or mitochondrial signaling pathway was showed.

3.6. Conclusion

Present study provides more evidence to support the anticancer effect of myricetin. Further, studies of myricetin *in vivo* are still necessary and should be the next recommended step. In addition, it should be considered that the absorbed concentration of myricetin must be lower than the intake concentration due to metabolisms factors. Moreover, it is also important to consider some factors in the cellular microenvironment of our human bodies such as pH and oxygen concentration. Generally, myricetin induced the apoptosis mediated by p53 activation in breast cancer MCF-7 cell lines.

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Chapter 3

3.1. General Discussion

Polyphenols have been widely studied for their antioxidant properties, especially preventing damage from reactive oxygen species (ROS) that formed as by-products of mitochondrial respiration or by certain oxidases or preventing generation of these species including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and hydroxyl radical ($\text{OH}\cdot$) (1). They are active in many cellular events, including as second messengers in the activation of several signaling pathways leading to the activation of transcription factors, mitogenesis, gene expression, and the trigger of apoptosis (2-4). Polyphenols show antioxidant property in various aspects such as anti-histamine (5), anti-inflammatory (6), antibacterial (7), and antiviral activities (8). However, it is more important to point out that polyphenols also exhibit its potent prooxidant property in lots of in vitro assays, anticancer effect in particular. In order to reduce the risk of chronic diseases have pointed much research to discern the lifestyle choices that potentially stimulate developing chronic pathologies, especially cancer. Epidemiological evidence has been already reported that the daily consumption of fruits and vegetables can reduce the risk of developing malignancies, especially, the polyphenol components of phytochemicals have been identified as anticarcinogens (9). Additionally, glutathione (γ -glutamylcysteinylglycine; GSH) plays a critical role in maintaining intracellular redox balance and alleviating ROS-induced oxidative stress. A major function of GSH is to scavenge ROS and thereby to prevent oxidative damage [10]. Because oxidative stress has been implicated with cancer, as well as with other chronic diseases and pathologies, including atherosclerosis, neurodegenerative diseases, and aging, much research has focused on the antioxidant properties of plant derived polyphenols. Interestingly, naturally occurring antioxidant polyphenols exhibit both pro-oxidative and antioxidative properties, depending on the factors as their metal-reducing potential, chelating behavior, pH, and solubility characteristics [11].

It was reported that either green tea or with red wine inhibited proliferation of rat pheochromocytoma PC12 cells through generating H_2O_2 . And the addition of catalase completely abolished the antiproliferative effects of green tea, but only partially reduced that of red wine. It was suggested that the toxicity of red wine was a combination of H_2O_2 with resveratrol, the main polyphenol in red wine, which has already known its antiproliferative effects [12]. Some researchers [13], using PC12 cells which derived from a pheochromocytoma of the rat adrenal medulla, showed that the level of EGCG was critical in evoking cell death by apoptosis. Low levels of EGCG (e.g., 50 μM) apparently induced mild oxidative stress, while a higher level EGCG (e.g., 400 μM) stimulated oxidative stress, as indicated by a persistent elevated intracellular level of ROS, induce disruption of the intracellular GSH level and an increase in lipid peroxidation. In the present study, myricetin showed no effect on the apoptosis in PC12 cells under 10% FBS condition. But enhansive effect was exhibited on the apoptosis induced by serum deprivation in PC12 cells. It is necessary to point out that the grace doses of myricetin (0.1 and 1 μM) were selected in this study.

Previously, Hsuuw and Chan [14] reported that the effect of EGCG with moderate (20–50 μM) and to high levels (100–400 μM) in human breast cancer MCF-7 cells. As a result, apoptosis was induced when MCF-7 exposed to moderate level of EGCG, while necrosis was induced when exposed to high level of EGCG. After MCF-7 cells being exposed to moderate levels of EGCG, cell viability was decreased, and apoptosis was induced. It is found a closed relation with increased oxidative stress, as indicated by intracellular generation of ROS, a loss of mitochondrial membrane potential, activation of caspase-3, caspase-9, and c-Junterminal kinase (JNK), and an increased expression level of Bax protein and a decreased level of Bcl-2 protein, finally shifting the Bax-Bcl-2 ratio to trigger apoptosis. However, little levels of ROS and of apoptotic cells and only minor effects on caspase activation, Bax-Bcl-2 ratio, and on mitochondrial membrane potential were showed. Cell death correlated with leakage of LDH, a sign of necrosis. Lowered ATP levels were observed at the high levels of EGCG. It was suggested that the switch from apoptotic death to necrosis was controlled by the intracellular level of ATP, with

high ATP levels favoring apoptosis and decreased levels favoring necrosis. Herein, myricetin showed an enhansive effect on the apoptosis induced by serum deprivation but not normal serum condition in PC12 cells, and moreover a potent toxicity on breast cancer MCF-7 cell lines which was also noted by the induction of apoptosis. It is possible to point out that the sensitivity of two cell line is different. However, both of the apoptosis mechanisms are consistent. For instant, in the MCF-7 experiment, cell viability results showed cell death induction in a dose dependent manner. Western blot results implied the trigger of apoptosis, which was induced by myricetin in a dose dependent manner. Our past study has already reported myricetin could enhance apoptosis induced by serum deprivation in PC12 cells mediated by mitochondrial signaling pathway (15). Serum starvation, as external stimuli, triggered the activation of p53. And p53-induced apoptosis in the way of regulating transcription of pro-apoptotic Bcl-2 family members like Bax which has been already investigated by Miyashita and Reed (16) previously. Moreover, p53 activation evokes a series of complicated and closed link to execute apoptosis by the release of cytochrome c. In fact, the release of cytochrome c, which is induced by the transcriptional activation of Bcl-2 family members like pro-apoptotic Bax and anti-apoptotic Bcl-2, triggers the downstream caspase cascade reaction and apoptogenic protein expression. Generally, the released cytosolic cytochrome c which binds with the adaptor molecular Apaf-1, forms a complex with oligomerized caspase 9 in the presence of ATP to activate caspase-3 and sequentially cleave cellular death substrates. Herein, a consistent mechanism of apoptotic signaling pathway was demonstrated. Myricetin, serve as toxic stimuli for MCF-7 cells, induced gradual cell death in a dose dependent manner which was shown from MTT assay and cell viability results. In this process, myricetin stimulated more ROS generation, which can be reflected by the lowering of GSH levels. The changes of GSH levels imply the imbalance of redox in intracellular environment. The overload of ROS triggered the activation of p53. Previous study already investigated the suppression of p53 activation could contribute in the role of NF- κ B in tumorigenesis and other diseases. In present study, the activation of p53 attenuated the role of NF-kB

contrarily and induced the transcriptional activation of Bax versus Bcl-2 dimers. Additionally, the protein expression of anti-apoptotic Bcl-x also showed a decreasing trend. Moreover, the downstream caspase cascade reaction was stimulated like caspase-3 protein expression. Indeed, myricetin regulated apoptosis related factors such as NF- κ B, p53, Bax, Bcl-2, Bcl-xL, cytochrome c, Apaf-1, caspase-3 and 9 in the intrinsic signaling pathway *in vitro*.

The locations of hydroxyl groups play important to the biological activities of flavonoid. Previous study reported hydroxyl groups at C3', C4' and C5' are important for the cytotoxic effect of myricetin on HL-60 human promyeloleukemic cells and THP-1 mature monocytic cells (17). If OH groups which located at C3', C4' and C5' were deleted such as galangin or replaced by OCH₃, both of their IC₅₀ were higher than myricetin (17). It demonstrated that the location of hydroxyl groups at C3', C4' and C5' exhibited higher level of toxicity. It was pointed out that the hydroxylation at C4' or C6 plays an essential role for apoptosis-inducing activity of flavanone through activation of caspase-3 cascade and production of reactive oxygen species (18). Large numbers of external stimuli induced apoptosis via ROS production although there was no ROS production determined by several ROS assay systems in Ching's study (17). However, ROS, function as mitogens, can also induce proliferation and protect cells from apoptosis induced by oxidative species (19, 20). These data indicates ROS exhibits double-sided fuction. In the present study, the redox balance was disrupted by myricetin according to GSH peroxidase into GSSG because of ROS production. On the contrary, most of flavonoids with multiple OH substitutions like quercetin have exhibited mutagenic *in vitro* through prooxidant rather than antioxidant action (21).

3.2 References

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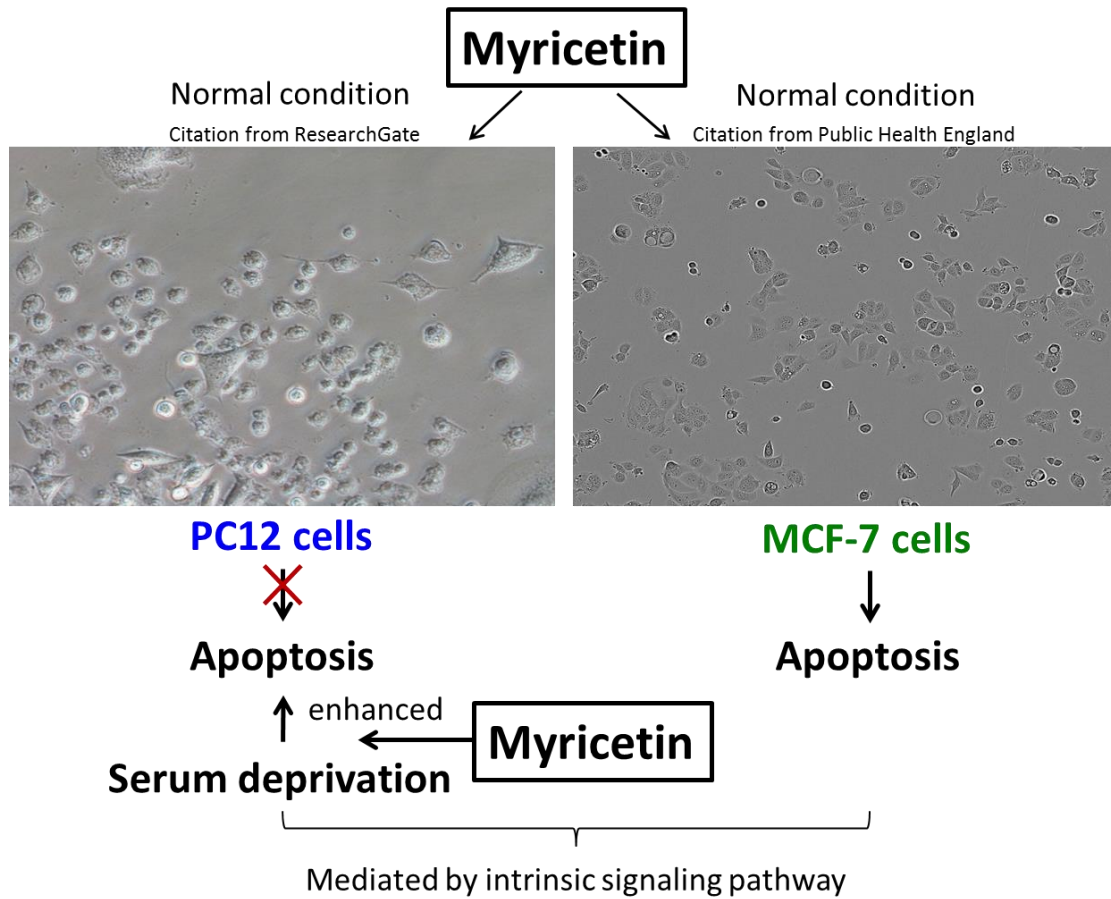
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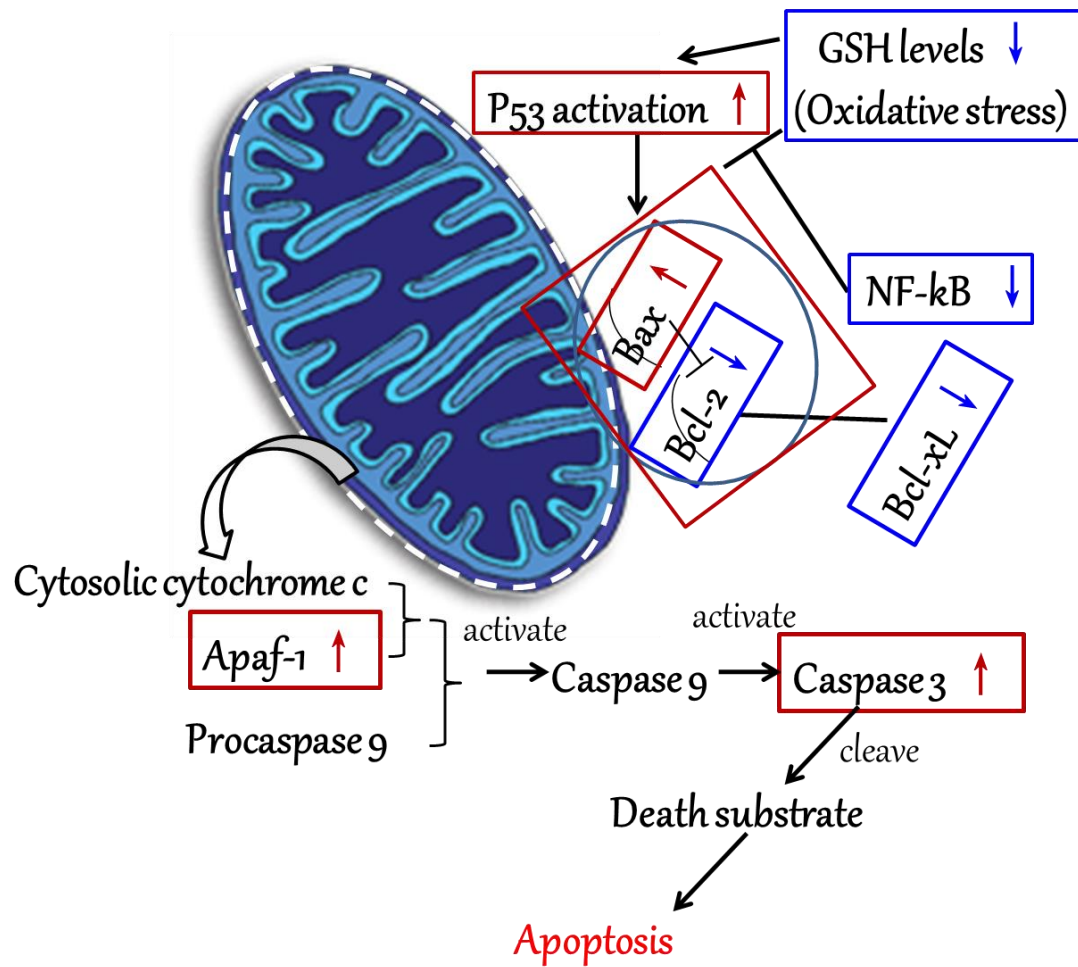
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Chapter 4

4.1. Conclusion

The present research exhibited the potent inhibitory effect of myricetin on the growth of PC12 cells and MCF-7 cells through apoptotic signaling pathways. Herein, the activation of p53 plays a critical role for triggering a series of molecular biological changes including disruption on the balance of intracellular redox environment and some apoptosis related proteins such as p53, NF- κ B, Bax, Bcl-2, cytochrome c, caspases-9 and 3. The anticancer mechanism of most anticancer drug or polyphenols depends on intrinsic signaling pathway as mentioned in the past chapter. Mitochondria played a central role in the intrinsic signaling pathway. The mitochondrial membrane potential causes increase of mitochondrial voltage-dependent anion channel (VDAC), loss of mitochondrial outer membrane potential and damage on PTP and finally opening of the permeability transition pore. This is an irreversible step toward apoptosis, which has been reported. Intermembranous mitochondrial protein cytochrome c was released from mitochondria into cytosol. Cytosolic cytochrome c, binded with Apaf-1, formed a complex with procaspase-9 to activate caspase-3. The disruption of mitochondrial membrane permeability was triggered by the transcriptional activation of Bax versus Bcl-2 dimers, which directly induced by p53 activation. DNA fragmentation of PC12 cells was determined implicating the occurrence of apoptosis. Herein, myricetin did not induce apoptosis under normal condition (10% FBS), but enhanced apoptosis induced by serum deprivation in PC12 cells. Furthermore, myricetin directly induced apoptosis in MCF-7 cell lines. It is possible that the sensitivity of PC12 cells is lower than MCF-7 cells.

It is well known that myricetin exhibited its potent anti-oxidant property to protect from ageing. However, more potential prooxidant function should be paid attention as the data shown in the present study. Further study is needed for clarifying the further application of myricetin *in vivo* and in clinical studies. It can be

predicted that the absorbed concentration of myricetin will be different with intake concentration due to its digestion and metabolism. Generally, this study provided more evidence for clarifying the prooxidant effect of myricetin through intrinsic signaling pathway.