Study on the tumor specific cytotoxicity of Kampo medicine and its mechanism of action

(漢方薬の腫瘍選択的細胞毒性及び作用機構に関する研究)

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by
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A C C E P T A N C E

The dissertation entitle **Study on the tumor specific cytotoxicity of Kampo Herbal medicine and its mechanism of action**here to attached, prepared and presented by **Siti Susanti** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy** is hereby accepted.

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Studies on the tumor specific cytotoxicity of Kampo medicine and its mechanism of action

Chemotherapy as one of therapeutic options for cancer, until recently still has the drawbacks of severe side effects and dose-limiting toxicity. Given the reality of chemotherapy are unsatisfactory, innovations for healing cancer disease with the low side effects always becomes an important and attractive goal along the journey of anticancer drug discovery. Some herbal extracts of Kampo medicine are believed to contain different chemopreventives or chemotherapeutics. However, the scientific basis for their modes of action is limited. Therefore, the identification of non-toxic chemotherapeutics from herbal medicines remains to be an attractive research theme to advance cancer treatments.

The first study evaluated the cytotoxicity profiles of 364 herbal plant extracts, using various cancer and normal cell lines. The screening found occurrence of A-549 (human lung adenocarcinoma) specific cytotoxicity in 9 species of herbal plants, especially in the extract of *Arctium lappa* L. Moreover, purification of the selective cytotoxicity in the extract of *Arctium lappa* L. resulted in the identification of arctigenin as tumor specific agent that showed cytotoxicity to lung cancer (A549), liver cancer (Hep-G2) and stomach cancer (KATO III) cells, while no cytotoxicity to several normal cell lines. Arctigenin specifically inhibited the proliferation of cancer cells, which might consequently lead to the induction of apoptosis. Thus, this study found that arctigenin was one of cancer specific phytochemicals, and responsible for the tumor selective cytotoxicity of the herbal medicine.

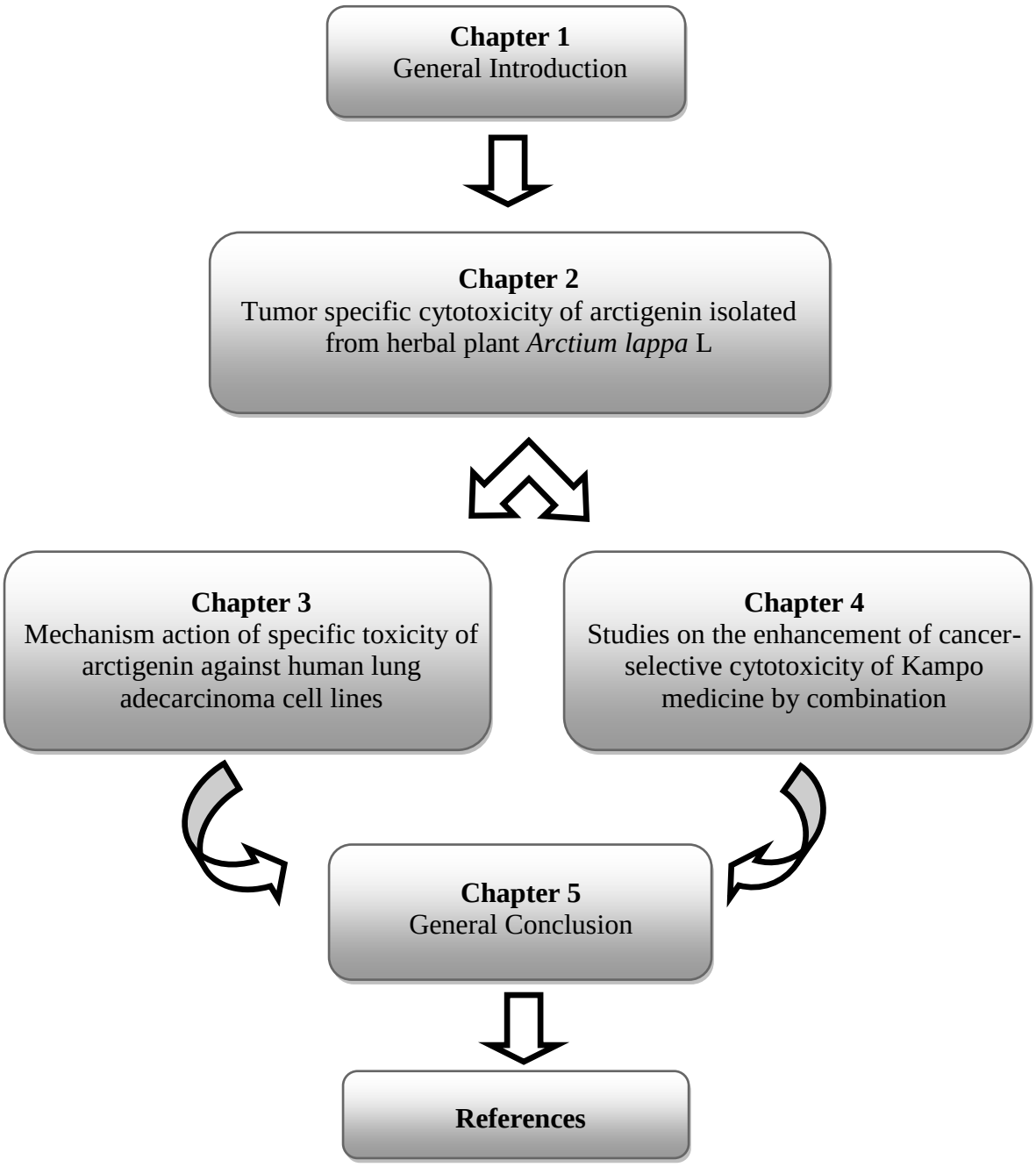
Next, the mechanism of action of arctigenin leading to specific cytotoxicity was studied. Arctigenin selectively arrested the cell cycle of cancer cells at G_0/G_1 phase, while induced apoptosis via modulation of gene expression in Akt-1 related signaling pathway. Furthermore, the cell cycle arrest in cancer cells at G_0/G_1 phase was found to be effected by the down-regulation of NPAT protein via the suppression of either cyclin E/CDK2 or cyclin H/CDK7. Furthermore, GSH synthase inhibitor specifically enhanced the cytotoxicity of arctigenin, suggesting that intracellular GSH content appeared to be another factor to influence the susceptibility of cancer cells to arctigenin.

Finally, we evaluated the selective cytotoxicity of 9 herbal plant extracts in single or mixed doses. Single and higher doses of herbal extracts selectively decreased the viability of cancer cells with slight toxicity to normal cells. A combination of lower doses of these extracts significantly increased the cytotoxicity to cancer cells with no adverse effect on normal cells suggesting that selectivity against cancer cells was enhanced and toxicity to normal cells was reduced by interactions between the wide arrays of compounds in the mixed formulations. We further tested for the positive or negative interactions between these crude extracts and arctigenin. On the basis of changes in ED_{50} values, it was found that a combination of arctigenin with extracts from *Prunus mume* or *Carum carvi* enhanced the cytotoxicity to cancer cells with no detrimental effect on normal cells. These observations indicated that combination of Kampo medicine can optimize cytotoxicity specifically to cancer cells while normal cells experience minimal toxicity.

In conclusion, this study clearly demonstrated that optimization of Kampo medicine can be achieved by appropriate combinations with respect to the specific cytotoxicity to cancer cells while ameliorating the toxicity to normal cells. Approaches based on interactions between Kampo-derived individual compounds or herbal plant

extracts are necessary to find the optimum Kampo formulation for cancer treatment, and may open a new avenue for cancer chemotherapy.

Layout and Flow of Dissertation



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Chapter 1
General Introduction

1.1. Background of study

1.1.1. Reality of cancer

The American Cancer Society (ACS) defines that cancer is a group of disease characterized by uncontrolled growth and spread of abnormal cells that can affect any other part of the body. Moreover, the World Health Organization (WHO) elaborates that cancer refers to the rapid creation of abnormal cells that grow beyond their usual boundaries. It may invade adjoining parts of the human body, spread to other organs, and quite possibly to cause death eventually. In both developed and the developing countries, cancer are the first or the second leading cause of death, respectively. (World Health Organization, 2008).

In the developing countries, the cancer case rises as a result of increase in population, human-aged, as well as adoption of cancer-associated lifestyles (e.g. smoking, physical-inactivity, "westernized" diets) (Jemal et al., 2011). It was estimated that there were ~12.7 million new cases of cancer and ~7.6 million deaths by cancer occurred in 2008; 56% of case and 63% of death occurred in the less developed regions of the world (Ferlay et al., 2010). The most diagnosed-cancers worldwide are lung, breast, and colorectal cancers, but lung, stomach and liver cancers are the most common killer disease to cause death. The details of estimated case of and death by cancers are listed in Table 1.1.

Table 1.1 Estimated new cancer case and death worldwide for leading cancer sites by level of economic development (2008).

No.	Cancer site	Estimated new case	Estimated death
	Male:		
1.	Lung and Bronchus	1,095,200	951,000
2.	Prostate	903,500	258,400
3.	Colon and Rectum	663,600	320,600
4.	Stomach	640,600	464,400
5.	Liver	522,400	478,300
6.	Esophagus	326,600	276,100
7.	Urinary bladder	297,300	112,300
8.	Non-Hodgkin Lymphoma	199,600	109,500
9.	Leukemia	195,900	143,700
10.	Oral cavity	170,900	*
11.	Pancreas	*	138,100
12.	All sites but skin	6,629,100	4,225,700
	Female:		
1.	Breast	1,383,500	458,400
2.	Colon and Rectum Prostate	570,100	288,100
3.	Cervix Uteri	529,800	275,100
4.	Lung and Bronchus	513,600	427,400
5.	Stomach	349,000	273,600
6.	Corpus uteri	287,100	*
7.	Liver	225,900	217,600
8.	Ovary	225,500	140,200
9.	Thyroid	163,000	*
10.	Non-Hodgkin Lymphoma	156,300	*
11.	Pancreas	*	127,900
12.	All sites but skin	6,038,400	3,345,800

* Not as leading cancer site
Source: GLOBOCAN (2008)

As the most common cancer throughout the world, lung cancer accounts for 13% of the total cases and 18% of all cancer-related deaths worldwide (Jemal et al., 2011). The high death rate associated with lung cancer is partially due to the fact that it is notoriously difficult to treat. For this reason, lung cancer survival rate is fairly low. Lung cancer is classified according to the histological type (Lu et al., 2010). This classification has important implication for clinical management and prognosis of the disease. The two broad classes are non-small cell (NSCLC) and small cell lung carcinoma (SCLC) (Maitra et al., 2007). Lung adenocarcinoma, the main sub-types of

NSCLC, is the most common form of lung cancer (Subramanian et al., 2007), and is likely associated with smoking habitual (Horn et al., 2012).

Taking above reality into consideration, finding a cure of cancer is always be an imperative endevour to conquer the disease. Until recently, innovation and development of anticancer agent is still being an interesting area to study by utilizing either natural or synthetic products. Each of these ingredients conceives some excellences and weaknesses, therefore related studies are of significantly required in order to obtain a promising anticancer agent in the future.

1.1.2. Dilemma of chemotherapy

The treatment of cancer is conducted through various ways such as chemotherapy, radiation therapy, surgery, immune activation, monoclonal antibody therapy and other methods (Skeel et al., 2003). The therapeutic method depends on the tumor location and stage of the development, as well as the general state of a personal health conditions.

Chemotherapy as one of therapeutic options for cancer still has the drawbacks of severe side effects and dose-limiting toxicity (Camp-Sorrell, 2000). The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body (Chabner and Longo, 2005). In most cases, target of anticancer agents is on the cells that divide rapidly. As mentioned before, the main feature of cancer is the rapid and uncontrolled growth of abnormal cells beyond their usual boundaries. However, in addition to malignant cancer cells, several cells divide very quickly under normal circumstances, such as cells of the bone marrow, digestive tract and hair follicles (Hirsch, 2006). This indicates that chemotherapy also harms these cells, which is generally called chemotherapy side effects: myelosuppression (decreased production of blood cells,

hence also immunosuppression), mucositis (inflammation of the lining of the digestive tract), and alopecia (hair loss) (Joensuu, 2008).

Given the reality that there is a relative unsatisfactory on chemotherapy, innovations for healing cancer disease with the low side effects always become an important and attractive goal along the journey of anticancer drug discovery. An ideal drug for cancer therapy is indispensable, wherein it is not only cytotoxic (i.e. able to kill cancer cells) but also has the ability to increase life quality of cancer patients. In this sense, the higher cytotoxicity is insufficient to fulfill the requirement as an anticancer treatment. The promising anticancer drug should have a feature of specific cytotoxicity so that an agent is only cytotoxic to certain cancer cells without giving adverse effects to normal cells.

1.1.3. Herbal plants as the one of natural product in the practice of traditional medicine system.

The role of natural products as source for remedies has been recognized since ancient times (Cragg et al., 1997). Moreover, natural product as a manifestation of nature's bounty for humankind has long been considered as a potential candidate of drug for prevention and treatment of cancer. Despite the progress reports in combinatorial chemistry and pharmacy, drugs derived from natural products still have an enormous contribution to drug discovery today (Balandrin et al., 1993; Cragg and Newman, 1999).

Herbal plants and their extracts are part of natural products which have been being trusted as the basic foundation of disease treatment for thousands years in the traditional medicine system. In addition, a wealth accumulation of clinical experience and indigenous knowledge of traditional medicine is still waiting to be tapped as an

important source for the modern drug discovery process. It has been recognized that utilization of herbal medicine may become a potential treatment in the future (Fabricant and Farnsworth, 2001). This is evidenced by the practises of traditional medicine that have been well accepted and trusted by the public until these days (Hoareau et al., 1999).

The traditional medicine systems that still exist today include Chinese Herbal Medicine (CHM), Kampo Medicine (KM) and Ayurvedic (AV). CHM is an ancient and complete medicinal system based on empirical observations, and has long been used for treatment of several kinds of diseases, especially for malignant cancer (Chia et al., 2010). Basic principle of CHM state that illness can be effectively treated by combination of herbal medicines based on their various characteristic and the overall status of patients (Ruan et al., 2006). On the other hand, KM consists of traditional medicine system adapted from CHM and is usually characterized by a variety of herbal plants formula that widely practiced in Japan, and is fully integrated into the modern health care system (Alphen et al., 1995).

Both CHM and KM derived from plant extracts are being increasingly utilized to treat a wide variety of illness because of such extracts are believed to contain different chemo-preventives or chemotherapeutics, which possess more than one mechanism of action. However, the scientific basis for their modes of action is limited (Cragg et al., 1997). Because of lack of experimental and clinical studies concerning the efficacy and safety, herbal medicine has not been fully accepted as cancer therapeutics (Buchanan et al., 2005). For this reason, in vitro study to evaluate their efficacy and safety is of quite necessary prior to the further test by either in vivo study or clinical trial.

1.2. Objective of study

It is now recognized that cancer is one of the leading killer disease. The fact that human with modern life style has potential health risk of cancer disease supports the view that incidence of cancer will increase continuously. In the meantime, dilemma of chemotherapy may hamper the development of anti-cancer agent because of a minimal expectation for survival elongation and improvement in symptom and quality of life.

This study attempts to furnish an answer to the chemotherapy dilemma by utilizing herbal plants listed in KM (either single or combination applications) to seek the potential anticancer agent possessing specific cytotoxicity against cancer cell. As one of natural products, herbal plants have formed the main basis for the treatment of disease in traditional medicine system reliable for its effectiveness and safety. Previous studies have reported the activities of herbal plant as an anticancer agent. Unfortunately, the scientific data of their activities and safety is yet to come.

In order to evaluate the specificity of herbal plants as anticancer agent, present study utilizes not only various cancer cells but also normal cells for a reference to demonstrate that the bioactive constituent in herbal plant extracts fulfill the safety requirement of anticancer agent with low side effect.

Based on above description, the following specific objectives have been worked out:

1. To carry out the screening cytotoxicity test toward 364 extracts of herbal plants mentioned in KM system by using various normal and cancer cell lines.
2. To isolate and purify an extract of herbal plant which possesses the most potent tumor specific cytotoxicity in order to get a bioactive constituent as its active ingredient.

3. To evaluate the further efficacy and safety of mixture formula of several extracts which perform the specific cytotoxicity resulted from the screening test experiment.
4. To find the best couple combination regarding the enhancement of tumor specific cytotoxicity between an active compound yielded from the purification process and each extract resulted from the screening test above.
5. To get more insight concerning the mechanism of tumor specific phytochemical which in part responsible for specific cytotocity of the herbal medicine.

1.3. Prospective of study

So far, as human being, we have merely been attracted by the modern and convenient life style, and become unconscious of that human is a part of nature. Under these circumstances, it is a time to go back to nature to solve the problems that have not been handled with the current human achievement called technology. Through this study on the tumor specific cytotoxicity of herbal medicine, it may remind us that nature with all of its bounty expressed as natural products offers a solution toward such problem related to human health disorder. In this case, when chemotherapy failed to solve a problem of cancer disease, utilizing some herbal plants as a manifestation of natural products inherited from traditional medicine may offer satisfying answer. By through evaluation of the potency of herbal plants, we are looking forward to an ideal medication that is not merely for the most potent in efficacy but also to the fulfillment of the bio-safety requirement.

According to the history, in the early of 16^{th} century, practices of Kampo medicine (KM) have been declined as a consequence of prevailed use of western medicine. Although the glory of KM returned back in the era from 1985 to 1995, however its application around the present period tends to decrease again (Fig. 1.1). The

data revealed from this study would give contribution toward the revival of KM. The entire suggestion of this study might arouse the people trust and interest toward KM practices integrated fully into the modern health care system.

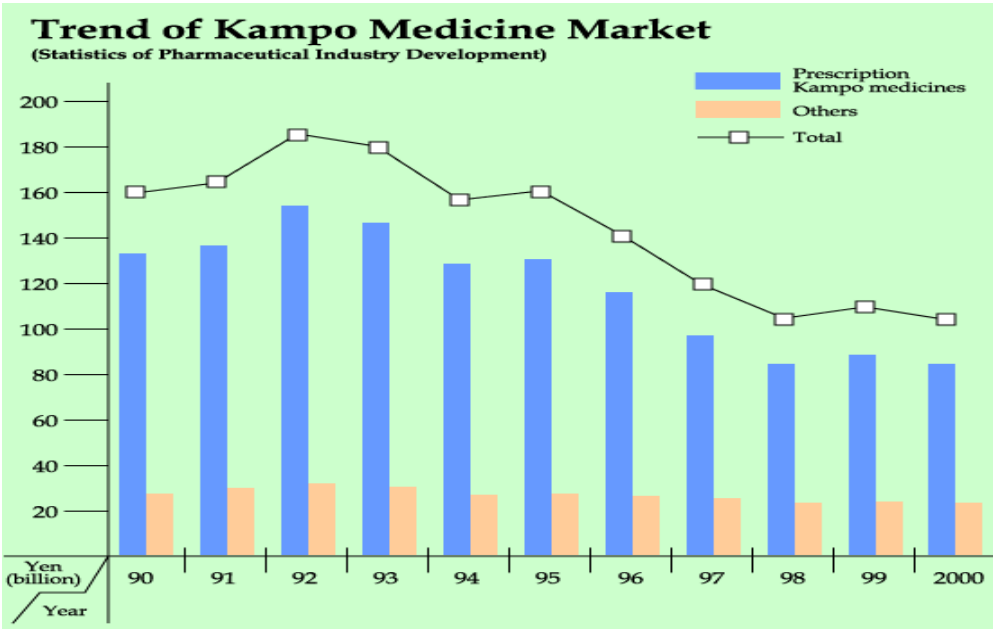


Fig. 1.1. Statistical graphic of trend of Kampo Medicine market in pharmaceutical industry development from years 1990 to 2000.

Chapter 2

Tumor specific cytotoxicity of arctigenin isolated from herbal plant *Arctium lappa* L

2.1. Introduction

Herbal medicine represents traditional medicines such as a variety of efficacious plants or plant extracts, which have been known to enhance healing of various diseases for thousands of years. The herbal medicines likely originated in the time of prehistoric era when people on all continents have used hundreds to thousands of indigenous plants for treatment of ailments. Currently, traditional use of medicines is recognized as a way to learn from ancient time about potential future medicine. In 2001, researchers identified 122 compounds derived from "ethnomedical" plant sources; 80% of these compounds were used in the same or related manner as the traditional ethnomedical use (Fabricant and Farnsworth, 2001).

One of the ethnomedical practices in the world that still exist today is Kampo medicine (KM). Japanese adopted the Chinese Herbal Medicine (CHM), and developed for over centuries as a unique KM system of diagnosis and treatment (Dharmananda, 2010). Although KM encompasses acupuncture, moxibustion, and other components of CHM, it relies primarily on prescription of herb formulas. The KM today manifests its primarily difference from the practice of CHM in the basic and primary herb collection of important herbs formulas (Terasawa, 2004).

According to Matsumoto (1999), there are three guiding principles in KM: simplicity, prevention and safety. KM consists of 365 herbal remedies that fall into three categories. The most highly valued remedies are the safety and prevention of disease. The prescriptions have the least side effects, if any, and intended for long-term use. The second class of remedies is to be used after an illness or medical problem and

is intended to revitalize the body and block recurrence of any health issues. The lowest level of remedy treats acute or chronic diseases and is only used for short intervals because of their potential toxic side effects. Interestingly, most Western allopathic medicines fall into this category. Another important feature of KM diagnostic methodology includes an analysis of an individual's personal constitution – how one's individual underlying nature predisposes one to specific health issues and treatments.

Recently, major worldwide health problem is occupied by lung cancer that explained approximately 16% of global cancer death (Pisani et al., 1999). The main types of lung cancer are *small-cell lung cancer* (SCLC) and *non-small-cell lung cancer* (NSCLC). Lung adenocarcinoma, a subgroup of NSCLC, account for approximately 75-85% of all lung cancers (Greenlee et al., 2001). Although chemotherapy is the standard treatment of NSCLC, its ability to improve the symptoms and the patient's quality of life only gives a minimal success and increase in survival rate (Ten et al., 1999). Given treatment outcomes using chemotherapeutic agents have been disappointing, further investigation is urgently needed to find a new therapeutic agents that possess highly toxic to lung adenocarcinoma, and little side effect on another normal tissue of the body.

Various plants extracts in the list of KM have been reported as chemo-preventives or chemotherapeutics agents (Tanaka et al., 2008; Kamaleeswari et al., 2006; Jeong et al., 2006; Kim et al., 2007). Along this decade, Burdock (*Arctium lappa L*) as one of herbal plant in KM is often studied about its antitumor activity. Arctigenin – a representative dibenzylbutyrolactone lignan as an active compound contained in this plant exhibited antitumor or antiproliferative activity in various cell cancers such as leukemia (Matsumoto et al., 2006), colon (Hausott et al., 2003; Yoo et al., 2010), pancreatic (Awale et al., 2006), pharynx (Jeong et al., 2011), and liver (Moritani et al., 1996) with various different mechanism of action. However, in the most of previous

study, effect of arctigenin to various normal cell still unclear until recently. Given such as the reality, herbal medicine likely has not been fully accepted as cancer therapeutics due to lack of experimental and clinical studies about its activity and safety (Buchanan et al., 2005). For this reason, it is necessary to study the specific cytotoxicity of arctigenin prior to further either invivo or clinical studies.

Although anticancer activity of arctigenin is quite promising, mechanism of its action on the lung adenocarcinoma has not been fully understood. As we know, activities of many cancer chemotherapeutic agents are effected by inhibition of cell proliferation by through an induction of cell death. There are two major mechanisms of cell death: necrosis and apoptosis. Cell damaged by external injury undergo necrosis, while cells undergo programmed death of apoptosis on internal or external stimuli (Ghobrial et al., 2005). Apoptosis takes place through two main pathways, extrinsic cytoplasmic pathways or intrinsic mitochondrial pathway (Hockenbery et al., 1990; Zapata et al., 2001). Both pathways involve the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell (Scaffidi et al., 1998). This is the reason why caspases are often called the final effector. It leads to execution of the death signal by the activation of a series of proteases termed caspases. There are twelve caspases, and some of them are involved in apoptosis (Thornberry et al., 1998). The caspases that have been well described are caspase-3, -6, -7, -8, and -9 (Mancini, et al., 1998).

At the beginning of study, we have screened the cytotoxicity of 365 extracts of herbal plants mentioned in KM system, using various normal and cancer cell lines. Furthermore, an active compound that posses the tumor specific cytotoxicity was purified from one of herbal plants and characterized for its selective cytotoxicity and mechanism of action.

2.2. Materials and Methods

2.2.1. Plant extracts preparation

This study used 364 species of herbal plants as listed in Kampo Medicine System. Air-dried whole plants were purchased from Kojima Kampo, Osaka, Japan. Frozen sample plant (0.5 gram) was powdered in liquid nitrogen, and then extracted with 10 ml of 50% methanol (MeOH) at room temperature for 48 h. The 50% MeOH extracts were filtered through a sterile filter ((Millex-LG <0.20μm) and stored in a refrigerator. The concentration of this preparation represents 100% extract through this study.

2.2.2. Cell culture

Various human normal or cancer cell lines used for the antitumor assay listed below were purchased from the Japan cancer research source bank (JCRB, Ibaraki, Japan). Normal cell lines: WI-38, lung normal diploid fibroblast; KMST-6, human fibroblast; OUMS-36T-2F and OUMS-36T-5F, normal human embryo fibroblast. Cancer cell lines: Hela, cervical cancer; VMRC-LCP, lung squamous cell carcinoma; ACC-MESO-4, malignant pleural mesothelioma; PC-14, RERF-LC-KJ, and A549, lung adenocarcinoma; MIA-PaCa-2, pancreatic cancer; Hep-G2, liver cancer; KATO III, stomach cancer; SKBR-3, breast cancer; A431, epidermoid carcinoma. Cells and sub culture were maintained according to the supplier's recommendation. Culture medium were DMEM, EMEM, and RPMI (medium according to the type of cell) supplemented with 10% FBS (Fetal Bovine Serum). Cells were cultured under humidified atmosphere of 5% CO_2 at 37^0C.

2.2.3. Cytotoxicity tests

The cytotoxicity of samples was determined by measuring the cell viability using MTS assay according to the manufacture's instruction (Appendix 1) Briefly, the sample or vehicle was added to the 96-well plate, and dried aseptically for 30 min. Cells cytotoxicity titration curve was constructed with serial dilution of sample in a 96-well microplate. Cell suspended in the appropriate medium was seeded at $1x10^3$ cells (100µl) per well and incubated in humidified atmosphere, 5% CO_2 at 37^oC for overnight. The cell viability after the treatment was determined by MTS assay kit, or by living cell number based on the measurement of DNA concentration.

In the later case, DNA concentration was measured by using the Quant-iT^{TM} dsDNA HS Assay Kits (Appendix 2) after 4, 8, 24 and 48 hours according to the manufacturer's procedures, and was converted into cell number based on the standard curve constructed with the DNA concentration in the known number of serially diluted cells: A549, WI-38, and OUMS-36T-5F. All the experiments were performed in triplicate, and the cell cytotoxicity was expressed as the relative viability or living cell number of the sample-treated cells against untreated controls.

2.2.4. Screening of crude extracts

Screening surveyed the tumor specific cytotoxicity of 364 herbal plant extracts using cancer and normal cells of lung: A549 and WI-38, respectively. The primary extract from each herbal plant was added to the culture with the final maximum concentration of 1% in the medium and the cytotoxicity test was conducted by using MTS assay (Appendix 1). The percentage (%) in this case indicates the concentration of the original crude extract in the culture medium on the basis of mixing ratio (v/v).

2.2.5 Purification of the tumor specific cytotoxicity

Two hundred g of dried seed powder of *Arctium lappa L* was extracted with 4 L of 70% ethanol (EtOH) at 30^{o}C for 24 h with shaking at 200 rpm. The extract was filtered with Kiriyama filter paper (no.5A, φ 95 mm) and concentrated to 250 ml by rotary evaporator. The specific cytotoxicity activity in the extract was purified by the combination of several chromatographies. Briefly, the crude extract was subjected to partition chromatography between ethylacetate and water. The ethylacetate phase was applied to silica gel column (300x50 mm,Yamazen) and eluted stepwise with the mixture of ethylacetate and methanol (MeOH). The most active fraction eluted by ethylacetate was further purified by low-pressure isocratic reversed phase chromatography on ODS-SM-50DM column (100x50 mm, Yamazen) with the mixture of MeOH and 0.1% formic acid as elution solvent. Eluent were fractionated in 20 ml, and the active fractions of number 3 – 10 were concentrated to appropriate volume, further purified by preparative reversed phase HPLC (cosmosil column:150x4.6mm, Nacalai Tesque, Kyoto, Japan) using the same eluent as used for the low-pressure reversed phase chromatography.

2.2.6. Identification of chemical structure

The chemical structure of the active compound was identified by interpretation of mass spectra (MS) and nuclear magnetic resonance (NMR) spectra. Electrospray ionization of MS was collected with ESI-MS (Esquire 3000 plus, Bruker Daltonics, Billerica, MA, USA), and NMR spectra were recorded with a JEOL α-500 spectrometer (JEOL, Tokyo, Japan) at 500 MHz (^{1}H) and 125 MHz (^{13}C).

2.2.7. ED_{50} Determination

Specific cytotoxicity of an active compound obtained by the purification of *Arctium lappa* extract was evaluated in various cancer or normal cell lines. Cell viability was determined by MTS assay as described in the earlier section. After 12 hours incubation, cells seeded in the microplate were treated with the active compound. Data was taken after 48 h treatment using microplate reader (BIO-RAD model 550, Hayward, CA, USA). ED_{50} (half effective dose) was defined as the dose required to inhibit cell viability by 50% after specified test duration, and determined by fitting the following formula to the titration curve: $y=\beta_3+ \beta_4/\{1+\exp(\beta_1+\beta_2 x)\}$ where y = cell viability, x = concentration of the test substance in the medium; $\beta_1-\beta_4$=constant. Fitting of the formula after transformation to the linear model was done by simple regression analysis program written by R Version 2.11.1 (Copyright (C) 2010, The R Foundation for Statistical Computing).

2.2.8. Caspase-3 activity assay

Cell lines were seeded in the 96-well microplate with the cell density of $2x10^5$ cell/well. Cells were treated with the test substances for 12 h. Caspase-3 activity was measured by using a caspase-3 fluorometric assay kit (Appendix 3)

2.2.9. Statistical analysis

The data were expressed as mean ± standard deviation. Statistical significance between pairs of mean was evaluated by Student's *t*-test. Value of $p < 0.05$ or $p < 0.01$ was the criteria for a statistical significance.

2.3. Results

2.3.1. Tumor specific cytotoxicity of crude extracts

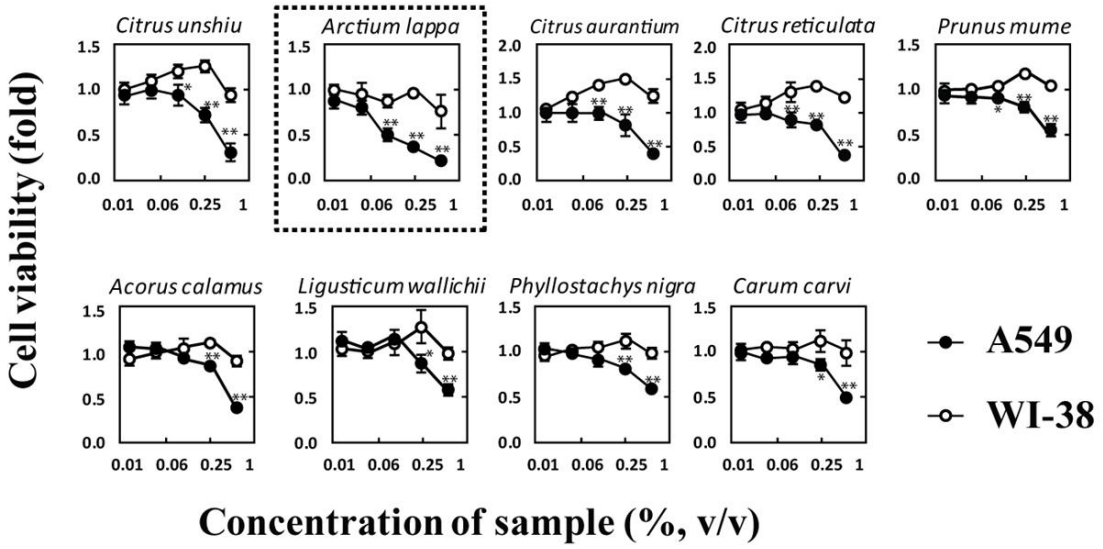


Fig. 2.1 Cell viabilities of A549 cells after exposure to the various concentration of herbal plant extract. Open and closed circle show the viabilities for normal and cancer cells, respectively. Cells seeded at density of 1,000 cells/well and pre-cultured in the medium for 24 h were treated with serially diluted 9 herbal extract for another 24 h. The viabilities were determined by MTS assay and expressed as the viability ratio of treated to untreated cells. Data are mean ±SE of triplicate analyses. *p<0.05; **p<0.01 versus the WI-38 cells

We screened 365 herbal plants from Japanese Kampo Medicine using pairs of cancer and normal cells established from lung: A549 and WI-38, respectively. This screening found that 9 specimens (*Citrus unshiu*, *Arctium lappa*, *Citrus aurantium*, *Citrus reticulate*, *Prunus mume*, *Acorus calamus*, *Ligusticum wallichii*, *Phyllostachys nigra*, and *Carum carvi*) inhibited the growth of human lung adenocarcinoma cell line (A549) without any adverse effect on lung normal cell line (WI-38). This observation indicates the occurrence of A549 specific cytotoxicity in these 9 extracts. The difference in the susceptibility between cancer and normal cells appeared to be most pronounced with the extract of *Arctium lappa*.L (Fig.2.1).

2.3.2. Purification and identification of specific cytotoxicity

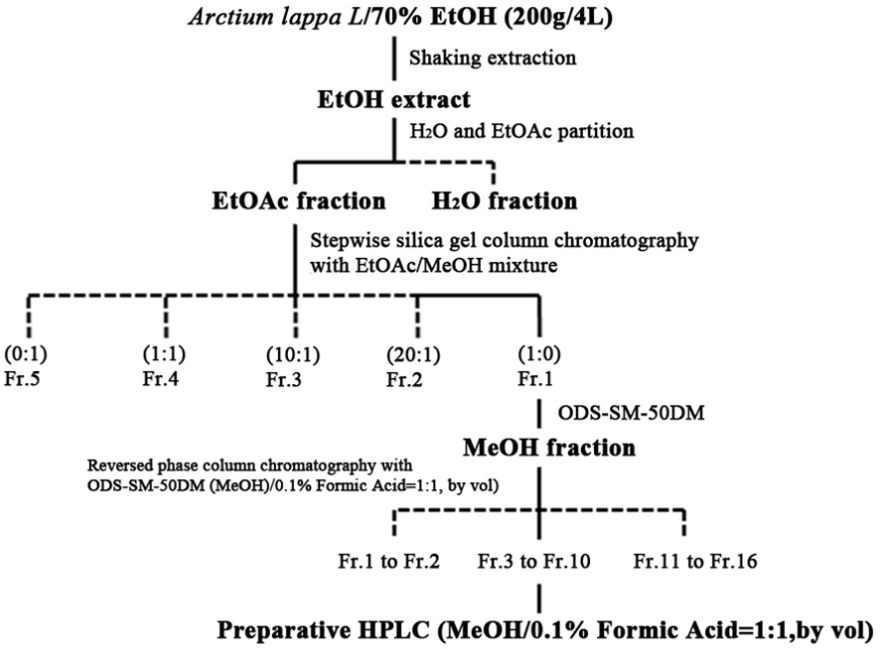


Fig. 2.2 The scheme of bioactivity-guided fractionation and purification of the tumor selective cytotoxicity from *Arctium lappa.* L.

In order to get more insight into the specific cytotoxicity, the authors purified the active compound in the extract of *Arctium lappa*.L. Purification process was described in the method section and was outlined in Fig. 2.2. The ethyl acetate (EtOAc) fraction (Fr. 1) on stepwise silica gel chromatography was found to have the most specific cytotoxicity among the others as shown in Fig. 2.3. Fr.1 was further subjected to purification on ODS-SM-50DM column (100x50 mm, Yamazen) with MeOH. Successive separation of this MeOH fraction on the same column with a mixture of methanol/0,1% formic acid (1/1, by vol.) result in the active fractions of Fr. 3–10 which was further purified by preparative HPLC (Shimadzu, Japan) on a Cosmosil column (150x4.6 mm, Nacalai Tesque, Kyoto, Japan). These process result in the purification of active component as depicted in Fig. 2.4: the largest peak eluted at 18 minute comprised

80% of total peak area, and was the component responsible for the the specific cytotoxicity in the extract of *Arctium lappa*.L (Fig. 2.4C).

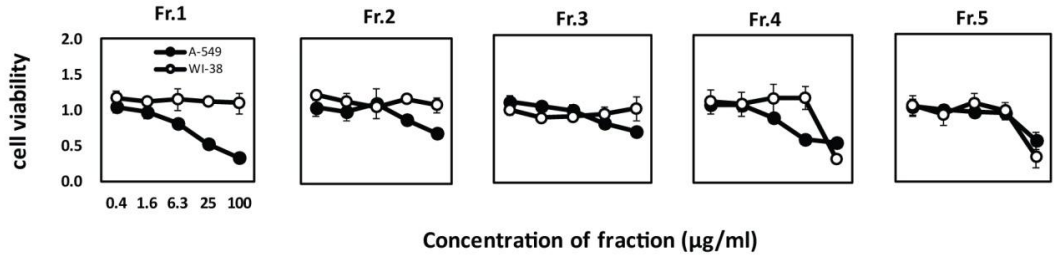


Fig. 2.3 Cytotoxicity test of the five fractions yielded from silica gel column operation to the lung adenocarcinoma (A549) cells and the lung normal diploid fibroblast (WI-38) cells

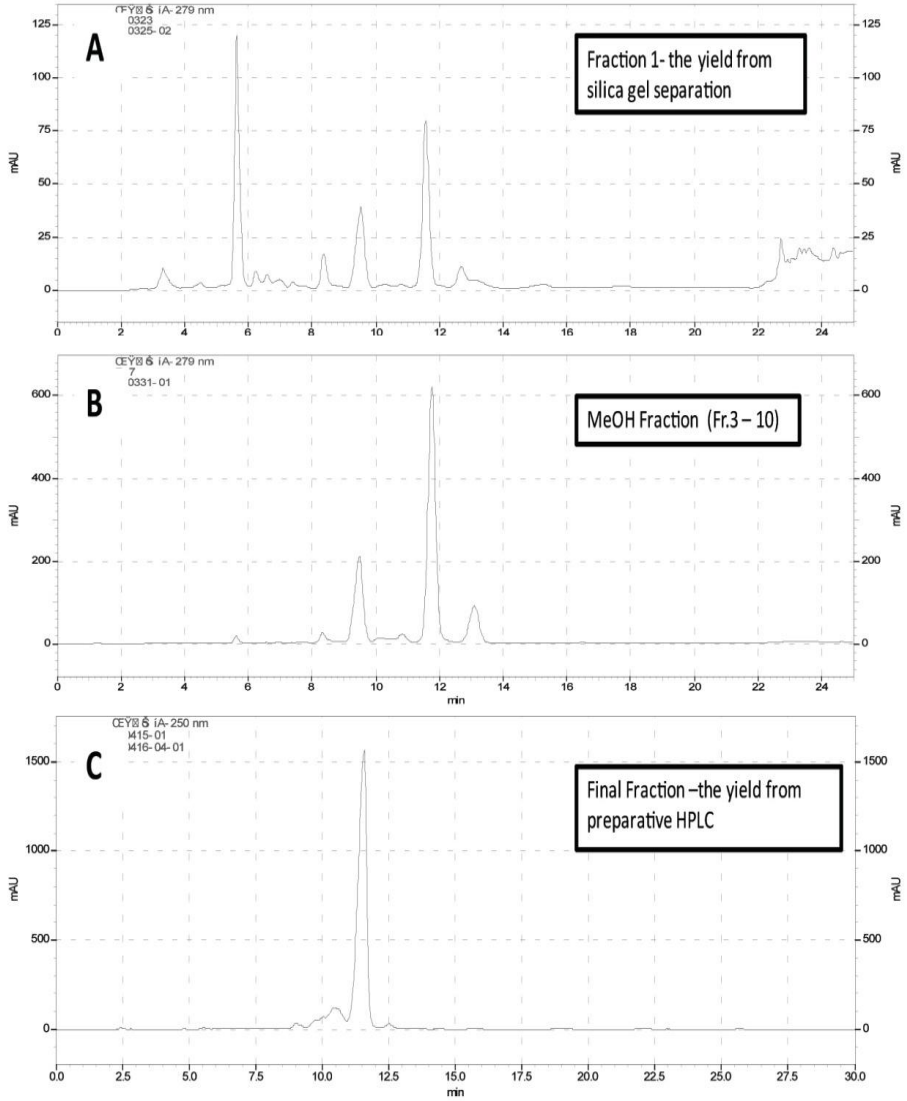
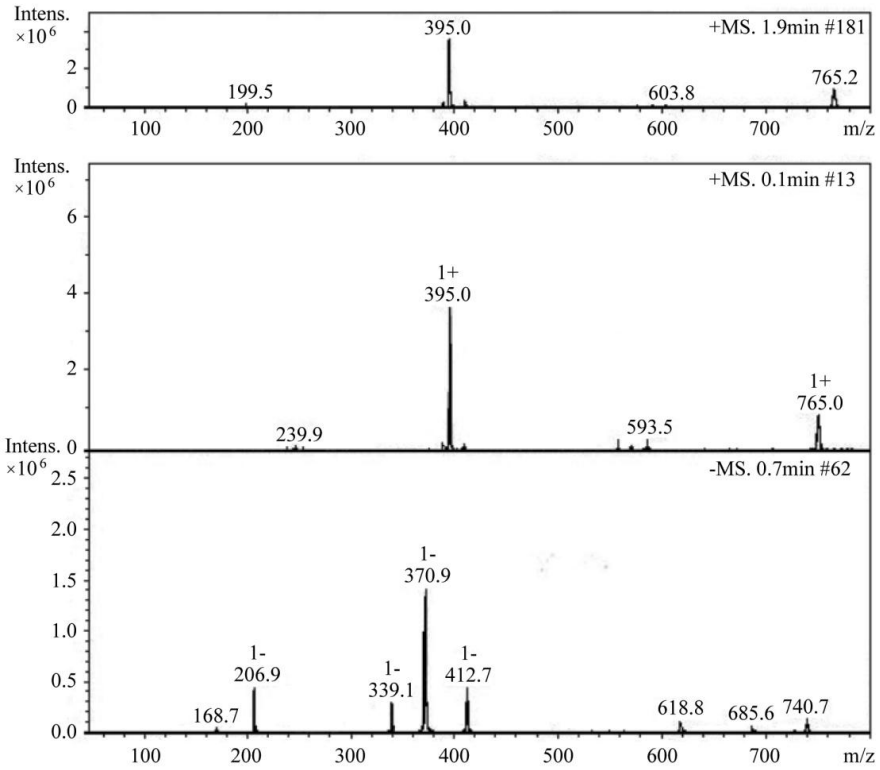


Fig. 2.4 HPLC analysis of some fractions and sub-fractions fractionated from Arctium lappa. L. extract.

The purified compound (4 mg) was dissolved in 400 µl of deuterated methanol [MeOH-d4] and applied to NMR spectrometer. ^{1}H and ^{13}C chemical shift are shown in Table 2.1. The molecular weight of the purified compound on ESI-MS was 395.0 with positive ion mode and 370.9 with negative ion mode (Fig. 2.5A). The comparison of NMR spectra with previous data (Umehara et al., 1993) identified the active material as arctigenin (IUPAC name: 3*R*,4*R*)-4-[(3,4-dimethoxyphenyl)methyl]-3-[(4-hydroxy-3-methoxyphenyl)methyl]-2-tetrahydrofuranone and molecular formula $C_{21}H_{24}O_6$.(Fig. 2.5B). The estimation of molecular weight by ESI-MS thus supported this identification.

A



B

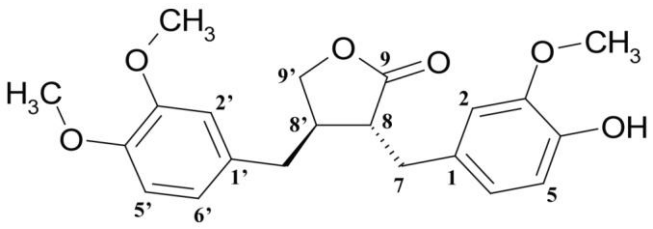


Fig. 2.5 Structure determination of the bioactive constituent having the tumor selective cytotoxicity: A, Mass spectra of the compound extracted from *Arctium lappa. L*; B, Based on the analysis of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) confirm its chemical structure to be arctigenin (molecular formula: $C_{21}H_{24}O_6$ and molar mass 372)

Table 2.1 ^{13}C and ^{1}H NMR spectral data of purified active material

Atom	^{13}C	^{1}H-NMRδ(mult,J(Hz),int)	HMBC correlations	HMQC correlations
1	130.751		H-5,H-7a	
2	113.851		H-7a,	H-2'
3	149.016	6.66(1H,d,J = 2.0Hz)	H-5,	
4	146.392		H-2,	
5	116.093	6.69(1H,d,J = 8.0Hz)		H-5
6	123.023	6.56(1H,dd,J = 2.0Hz,8.0Hz)	H-2,H-7a,H-7b	H-6
7a	35.417	2.88(1H,dd,J = 5.3Hz,14.0Hz)	H-8	H-7a,H-7b
7b	_a	2.80(1H,dd,J = 7.3Hz,13.9Hz)		
8	47.78	2.65(1H,m)		H-8
9	181.623		H-9'	
3-OCH_3	56.484	3.78(3H,s,OCH3)		
1'	132.855			
2'	122.074	6.59(1H,s)		H-2'
3'	150.454			
4'	149.135			
5'	113.04	6.80(1H,d,J = 8.0Hz)		H-5'
6'	113.607	6.59(1H,dd,J = 2.0Hz,8.0Hz)		H-6'
7'a	38.878	2.55(1H,s)		
7'b	_a	2.5(1H,dd,8.0Hz,14Hz)		
8'	42.446	2.54(1H,s)	H-7'b,H-8	
9'a	72.942	3.93(1H,dd,J = 7.6Hz,9.0Hz)	H-7'b,H-8'	H-9'a,H-9'b
9'b	_a	4.16(1H,dd,J = 7.5Hz,8.9Hz)		
3'-OCH_3	56.365	3.77(3H,s,OCH3)		
4'-OCH_3	56.339	3.75(3H,s,OCH3)		

Analysis conditions were 125 (^{13}C; 24^0C) and 500 (^{1}H; 30^0C) MHz, in MeOH-d4
[a]Signal could not be determined

2.3.3. Specific cytotoxicity of arctigenin

The specific cytotoxicity of arctigenin was evaluated by using various normal and cancer cell lines (Table 2.2). The ED_{50} defines the dose required to kill half of cell population after specified treatment and was determined as described in the method section. It was found that normal cell lines were less susceptible to arctigenin with ED_{50} value more than 100 μg/mL whereas the cancer cells are sensitive to arctigenin with ED_{50} value of 4.5, 5.4, 11,0 for cells HepG2, A549 and KATO III, respectively. It is

further noted that ED_{50} of arctigenin for cancer cell (A549) decreased with increasing treatment time to 72 h, while little change was seen for ED_{50} of normal cells up to 72 h of treatment (Fig. 2.6)

Table 2.2. Cytotoxicity (ED_{50}) of arctigenin

Cell type	Names	Descriptions	ED_{50} (μg/ml)
Normal	OUMS-36T-5F	normal human embryo fibroblast cell tranfected by hTRT gene	>100
	KMST-6	nomal human fibroblast cell	>100
	WI-38	lung normal diploid fibroblast	>100
Tumor	A549	lung adenocarcinoma cell line	5.4
	PC-14	lung adenocarcinoma cell line	>100
	RERF-LC-KJ	lung adenocarcinoma cell line	>100
	ACC-MESO-4	malignant pleural mesothelioma cell line	94.5
	VMRC-LCP	lung squamous cell carcinoma	62.0
	A431	epidermoid carcinoma	49.8
	KATOIII	stomach cancer (signet ring cell carcinoma)	11.0
	SKBR-3	breast carcinoma cell line	>100
	Hela	human epithelial cervical cancer	>100
	HepG2	human liver carcinoma cell line	4.5
	MIA PaCa-2	human pancreatic carcinoma cell line	>100

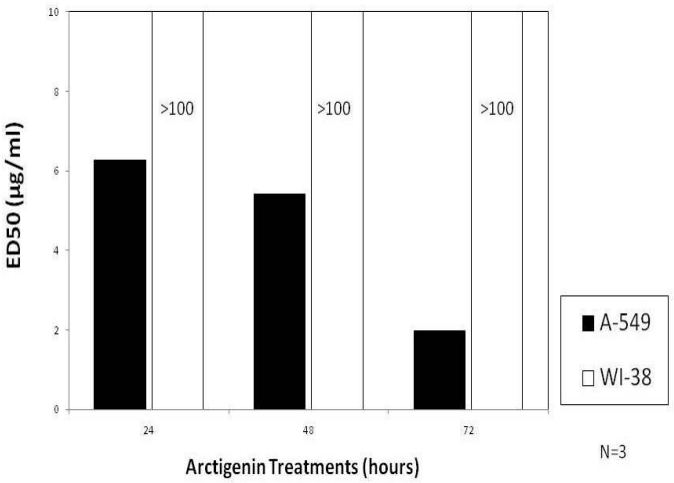


Fig. 2.6 Specific cytotoxicity of arctigenin to lung adenocarcinoma (A549) cells and the lung normal diploid fibroblast (WI-38) cells along the treatment time.

2.3.4. Effect of arctigenin on cell growth

To get more insight into the action mechanism of arctigenin, its effect on the cell growth was studied with cancer (A549) and normal cell lines (WI38 and OUMS-36T-5F). We experienced that MTS measurement was not necessarily a good hallmark for the cell growth because the measurement is an indicator of metabolically active mitochondria, and easily modulated by the metabolic status of cells (Wang et al., 2010). For this reason, this experiment used the DNA-based method to measure the cell growth.

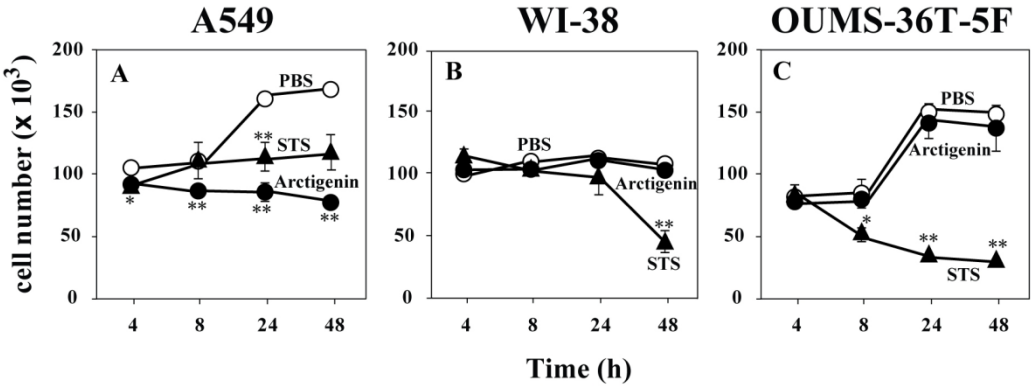


Fig. 2.7 Cell growth of A549 (A) and WI-38 (B) and OUMS-36T-5F (C) cells after exposure to arctigenin or staurosporin (STS). Cells seeded at density of 10^5 cells/well and pre-cultured in the medium for 24 h were treated with 50 μg/ml of arctigenin and 10μM of STS for 4, 8, 24 and 48 h. Cell numbers were determined by the DNA concentration as described in method section. Data are mean ±SE of triplicate analyses. *p<0.05; **p<0.01 versus the control (PBS)

The living cell number of non-treated control cancer cell (A549) increased with incubation time as shown in Fig. 2.7A. In contrast, cell number of A549 incubated with arctigenin slightly decreased with the treatment time. staurosporine (STS), a positive apoptosis inducer, also suppressed the cell growth, but to a lesser extent compared to arctigenin. The cell number of normal cells (WI-38) showed no changes with or without treatment of arctigenin, and decreased by STS toward 48 h of treatment (Fig. 2.7B). The growth of another type of normal cell (OUMS-36T-5F) depicted the same shape of

curve as illustrated for A549, and was also not influenced by arctigenin, but significantly lowered by STS (Fig. 2.7C).

2.3.5. Detection of caspase-3 activity

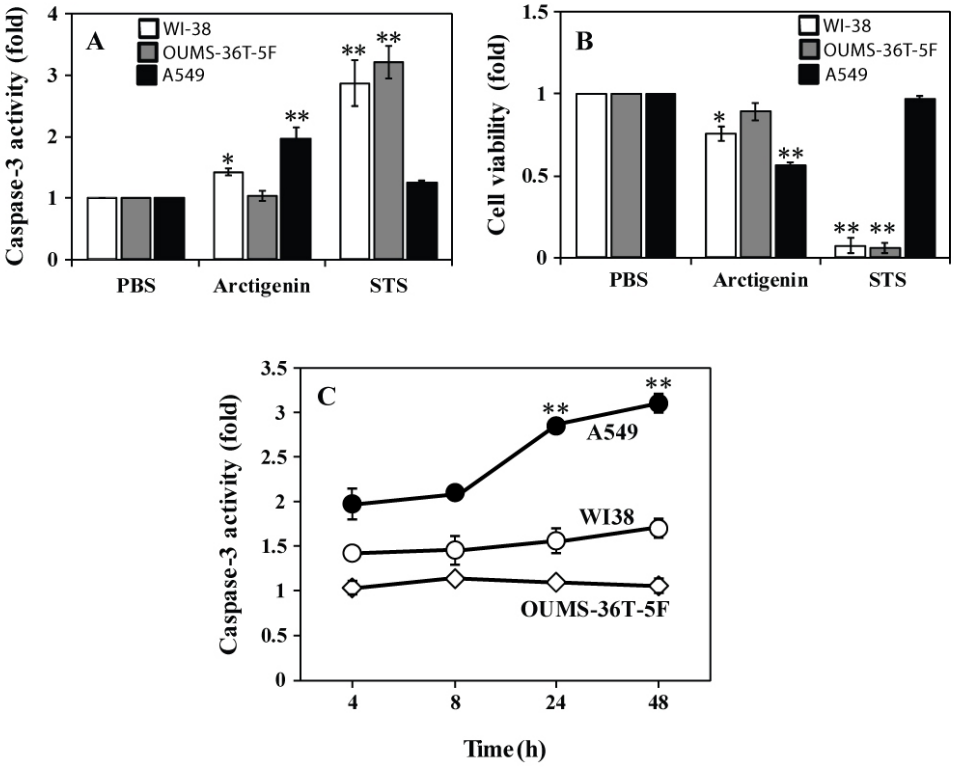


Fig. 2.8 Caspase-3 activities (A and C) and cell viabilities (B) of WI-38, OUMS-36T-5F and A549 cells after exposure to arctigenin (50 μg/ml) or staurosporin (STS, 10μM). Cells seeded at density of 100,000 cells/well and pre-cultured in the medium for 24 h were exposed to arctigenin or STS for 4 h (A and B), and for arctigenin for variable time (C). Cell viability was determined by MTS assay. Values were the ratio of the measurement of treated to that of untreated cells. Data are mean ±SE of triplicate analyses. *p<0.05; **p<0.01 versus untreated cell (A and B). **p<0.01 versus 4 h treatment (C)

To examine whether apoptosis is involved in the cytotoxicity of arctigenin, we measured caspase-3 activity in A549, WI-38 and OUMS-36T-5F cells after incubation for 4 h with arctigenin or positive control of staurosporine (STS). Arctigenin significantly enhanced caspase-3 activity of cancer A549 cells to almost 2-fold of

control, and also slightly increased that of normal WI38 cells (Fig. 2.8A). STS activated the caspase-3 activity of normal cells rather than the cancer cells to much greater extent compared with the case for arctigenin (Fig.4A). Activation of caspase-3 by arctigenin or staurosporine correlated with the decrease in the viability of both cancer and normal cells (Fig. 2.8B). Furthermore, caspase-3 activity significantly increased with increasing treatment time only in A549 cancer cells, but not in WI-38 and OUMS-36T-5F normal cells (Fig. 2.8C).

2.4. Discussion

Present study demonstrated the tumor specific cytotoxicity of herbal plants for the first time. This study conducted the screening of a number of herbal plants of Kampo medicine (KM), isolation of the active component from crude extracts and its cytotoxicity test. Several previous studies have demonstrated the anti-cancer activity of herbal plant extracts (Chia et al., 2010; Kumar et al., 2004; Park et al., 2011; Ranga et al., 2005). However, the tumor specific cytotoxicity of those extracts has not yet been reported. The screening of current study informed us that several herbal plants extract (especially *Arctium lappa.* L extract) have potential for chemopreventive or chemotherapeutic agent with low side effects due to their cancer specific cytotoxicity (Fig. 2.1). Previous studies suggested that flavanoids contents and their derivatives in these extracts were associated with anticancer activity (Kohno et al., 2001; Tanaka et al., 2008; Murakami et al., 2000; Tanaka et al., 2000; Garcia et al., 1997)

Purification of specific cytotoxicity in the extract of *Arctium lappa.* L.resulted in the identification of arctigenin as active component (Fig.2.5B). Arctigenin is a phenylpropanoid dibenzylbutyrolactone lignan with antioxidant, anti-inflammatory and antitumor activities (Awale et al., 2006; Cho et al., 2004; Tamayo et al., 2000; Zhao et

al., 2009). It has been shown that arctigenin inhibited the growth of various cancer cells: gastric (Jeong et al., 2011), liver (Moritani et al., 1996), colon (Hausott et al., 2003; Yoo et al., 2010), starved pancreas (Awale et al., 2006) and leukocyte (Matsumoto et al., 2006). Thus, arctigenin suppressed the proliferation of cancer cells by induction of apoptosis (Matsumoto et al., 2006) or by cell cycle arrest (Jeong et al., 2011; Hausott et al., 2003; Yoo et al., 2010). These previous studies, however, shed no light on the cancer specific cytotoxicity of arctigenin as shown in Table 2.2 in this study using various cancer cell panels. This beneficial feature of arctigenin is very important, and merit further investigation to develop the anti-cancer agent of low side effect. Exploring the mechanism underlying the specificity may open up another possibility for novel weapons of anti-cancer agent to attack specifically the cancer cells. Furthermore, this study demonstrated the specific cytotoxicity of arctigenin against lung cancer cells for the first time, in addition to the cancer cell lines studied hitherto.

The specific cytotoxiciy of arctigenin appeared to be mediated by inhibition of cell proliferation but not via the direct induction of apoptosis. Caspase-3 activity of A549 cancer cells increased with progression of arctigenin treatment as shown in Fig. 2.8C. However, the number of cells decreased gradually with the treatment time (Fig. 2.7A). This is in contrast to the observation for normal cell incubated with STS. STS is a potent inhibitor of PKC and many other kinases, and directly inhibits the activity of topoisomerase II (Bertrand et al., 1994; Wang et al., 2009). It has been reported that STS can induce apoptosis of a variety of cells (Entschladen et al., 2005; Zhang et al., 2005). Thus, STS induced apoptosis and activated caspase-3 to much greater extent in both OUMS-36T-5F and WI38 normal cells (Fig. 2.8A), leading to the rapid decrease in the living cell number (Figs. 2.7B and 2.7C). Given that the induction of apoptosis is the primarily and principal mechanism for the cancer selective cytotoxicity of arctigenin,

there should be a rapid and larger extent of increase in the caspase-3 activity as was the case for the normal cells on apoptosis induction by STS. Thus, present finding suggested that the inhibition of proliferation is primarily involved in the anti-cancer mechanism of arctigenin. It is well accepted that cell cycle arrest leads to the induction of apoptosis (Sherr, 1994). It is therefore likely that arctigenin specifically inhibited the cancer cell proliferation, and secondly induced apoptosis. Alternatively, it is also likely that arctigenin activate the death signaling of apoptosis to much lower extent than the other apoptotic anti-cancer agent.

STS in current study induced apoptosis in normal cells rather than the cancer cells, and decreased the cell number and the viability (Figs. 2.7 and 2.8). It has been reported that long-term exposure to STS was necessary for the effective growth inhibition of cancer cells (Courage et al., 1996). STS and its derivatives were found to be less potent with the exposure time shorter than 6 h (Wang et al., 2009). The treatment time of 4h in this study therefore may be too short to induce apoptosis in A549 cancer cells. In this context, it is worth noting that arctigenin was more potent to cancer cells even with short exposure time, and induced apoptosis without any cytotoxicity to normal cells (Figs. 2.7 and 2.8). This feature entirely contrasted with the case of STS, and may manifest the usefulness of this agent for the development of novel anti-cancer agent of low side effect.

Although anticancer activity of arctigenin appeared to be promising, the mechanism of its action has not been fully understood. Several studies reported that arctigenin blocks the unfolded protein response and the activation of Akt in glucose-deprived solid tumor (Awale et al., 2006; Kim et al., 2010). However, the mechanism proposed for the glucose-deprived cancer cells may not necessarily be applicable to our current study. Given that inhibition of cell proliferation is involved in the anti-cancer

mechanism of arctigenin, the signal transduction pathways leading to cell cycle arrest is entirely unknown. It has been demonstrated that arctigenin induced the cell cycle arrest at G_0/G_1 phase by modulation of the regulatory proteins such as Rb and cyclin D1 in gastric cancer cells (Jeong et al., 2011), and at G_2/M phase in colorectal cancer cells via regulation of Wnt/β-catenin signaling pathway (Yoo et al., 2010). Therefore, the cell cycle arrest point in arctigenin treated cells showed variation between cell types. It is also noteworthy that arctigenin was detoxified by glutathione (GSH) in liver cells (Moritani et al., 1996). Thus the expression level of GSH synthase also appeared to be a factor controlling the susceptibility of the cells to this agent (Moritani et al., 1996). Further studies are needed to understand the mechanism underlying the specific cytotoxicity of arctigenin in relation to the signal transduction or its stability as anti-cancer agent.

2.5. Conclusion

Arctigenin as tumor specific agent that showed cytotoxicity to lung cancer (A549), liver cancer (Hep-G2) and stomach cancer (KATO III) cells, while no cytotoxicity to several normal cell lines. It specifically inhibited the proliferation of cancer cells, which might consequently lead to the induction of apoptosis. This study found that arctigenin was one of cancer specific phytochemicals, and in part responsible for the tumor selective cytotoxicity of the herbal medicine.

Chapter 3

Mechanism action of specific toxicity of arctigenin against human lung adenocarcinoma cell lines

3.1. Introduction

Lung cancer is a major worldwide health problem and has accounted for approximately 16% of global cancer deaths (Pisani et al., 1999). There are two main types of lung cancer, i.e. small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). Lung adenocarcinoma, which belongs to NSCLC, comprised approximately 75-85% of all lung cancers (Greenlee et al., 2001).

Chemotherapy is known as a standard treatment for NSCLC. Despite the successful application of chemotherapy, its ability to improve the symptoms and the life quality of the patient only gives a minimum achievement (Ten et al., 1999). Some therapies for lung cancer have been the promising strategy to control the disease progression. Nevertheless, the toxicity of these therapeutics restrict the completion of the recommended dose. Therefore, an anticancer agent of high selectivity against NSCLC merits further investigation rather than to search for agents of high toxicity.

There is a growing attention to the pharmaceutical value and the biological activity of herbal plant medicine since last decade. Numerous bioactive compounds from herbal plants exhibit selective toxicity toward tumorigenic tissues, and thus display cancer-targeting properties (Quasney et al., 2001). One group of chemical compounds found in plants is lignan. Arctigenin (i.e. a representative of dibenzylbutyrolactone lignan isolated from *Arctium lappa* L.) has been reported to possess a number of important pharmacological or biological activities of anti-oxidation, anti-tumorgenesis and anti-inflammation (Awale et al., 2006; Matsumoto et al., 2006). Our previous study

demonstrated that arctigenin specifically inhibited the proliferation of lung adenocarcinoma (A549) cells, which might consequently lead to the induction of apoptosis (Susanti et al., 2012). Apoptosis, or programmed cell death, has been known as the major mechanism to counter act with the DNA damage. Apoptosis also is important to control cell number and proliferation as a part of normal development (Lowe et al., 2000). Thus, the understanding of underlying mechanism of apoptosis provides important information to find a novel cancer targeting therapies or to sensitize them against chemotherapeutics (Ghobrial et al., 2010).

In addition to apoptosis, modulation of cell cylce explains the survival or non-survival process of cell. The cell cycle, or cell-division cycle, is the series of events that take a place in a cell leading to its division and duplication (replication). The cell cycle consists of four distinct phases: G_1 phase, S phase (synthesis), G_2 phase (collectively known as interphase) and M phase (mitosis). Activation of each phase is dependent on the proper progression and completion of the previous step. Regulation of the cell cycle involves the detection and repair of DNA damage as well as the prevention of uncontrolled cell division. Two key classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), play a pivotal role in the completion of the cell cycle (Nigg et al., 1995).

A number of studies have reported the anti-cancer mechanism of arctigenin in several types of cancer cells. For example, arctigenin was proven to inhibit the cell growth in colon cancer via the Wnt/β-catenin (Yoo et al., 2010). Arctigenin also induces cell cycle arrest by blocking the phosphorylation of Rb in human gastric cancer cells (Jeong et al., 2011). Despite the noteworthy conclusions in such previous studies, the mechanism responsible for the specific cytotoxicity of arctigenin in lung cancer cell has remained un-elucidated. The current study aims to get more insight into the mechanism

of arctigenin to inhibit proliferation and to induce and apoptosis in lung adenocarcinoma. Furthermore, this study also aims to delineate the specificity of arctigenin by comparative studies on various normal cell lines. The results of this study will provide valuable new information on the usefulness of arctigenin as a promising chemotherapeutic agent of low side effect.

3.2. Materials and methods

3.2.1. Isolation and purification of arctigenin

Isolation and purification of arctigenin from *Arctium lappa* L. extract was performed following the method as described previosly in Susanti et al. (2012).

3.2.2. Cell cultures

Various human normal embryo fibroblast (OUMS-36, OUMS-36T-2F and OUMS-36T-5F) and lung adenocarcinoma (A549) cell lines were purchased from the Japanese Cancer Research Resources Bank (JCRB, Ibaraki, Japan). Cells were cultured in DMEM supplemented with 10% FBS (Fetal Bovine Serum) and at 37°C in a humidified 5% CO_2 atmosphere.

3.2.3. Cell viability assay

Cells suspended in DMEM were seeded at 1×10^4 cells (100 μl) per well of 96-well plates and pre-incubated overnight in humidified atmosphere of 5% CO_2 at 37°C. After 24 h incubation with the varying concentration of arctigenin, the cell viability was determined by MTS assay kit (Appendix 1). All the experiments were performed

triplicate, and the cell viability is expressed as the relative viability of the sample-treated cells against untreated cells (control).

3.2.4. Cell cycle analysis

Cells (3×10^5) were seeded in 60 mm culture dishes and pre-incubated for 24 h at 37°C. Cells were washed with PBS before medium replacement. Cells were cultured in DMEM supplemented with (6 μg/ml) or without arctigenin. After 24 h incubation, cells were harvested by using Accutase® Cell Detachment Solution, washed with PBS, fixed with 70% cold ethanol and incubated overnight at 4°C. Ethanol was decanted off, and cells were re-suspended in PBS on ice for 10 min. Cell suspensions were centrifuged at 1000 rpm for 10 min, re-suspended with 250 U/ml RNase solutions and incubated for 20 min at room temperature. After adding of 200 μg/ml propidium iodide solution, cell suspensions were transferred and filtered through a 35 mM nylon filter into flow cytometer tubes. Data acquisition and analysis were performed by FACS Calibur flowcytometer system (BD Biosciences).

3.2.5. Total RNA isolation and quantitative real-time PCR analysis

Isolation of total RNA and PCR analysis were performed following the manufacturer's instructions (Appendices 4–7). Total RNA was extracted from either untreated or treated cells (1×10^6) using the AquaPure RNA Isolation Kit (Bio-Rad Laboratories) (Appendix 4). The quality of RNA was checked using MultiNA Reagent Kit on microchip electrophoresis system (MultiNA-Biotech, Shimadzu, Japan) (Appendix 5). Total RNA (2μg) was reverse-transcribed to produce cDNA by using High Capacity RNA-to-cDNA Kit (Appendix 6).

Table 3.1 Primer sequences used for quantitative real time RT-PCR

Gene	Description	Primer sequence	Product	Accession No.
Fas	Fas (TNF receptor superfamily, member 6)	5'-AAGAATGGTGTCAATGAAGCCA-3' (forward primer, F) 5'-GAAGTTGATGCCAATTACGAAGC-3' (reverse primer, R)	103	NM_152871.2
Bcl-2	B-cell lymphoma 2	5'-TTCTACGACAGCAAATTGCCC-3' (forward primer, F) 5'-TTGCCTTATCCATTCTCCTGTGT-3' (reverse primer, R)	102	NM_004049.3
Bax	BCL2-associated X protein	5'-TCTGACGGCAACTTCAACTGG-3' (forward primer, F) 5'-AGCCCATGATGGTTCTGATCA-3' (reverse primer, R)	115	NM_138764.4
Bad	BCL2-associated agonist of cell death	5'-CAGTGACCTTCGCTCCACATC-3' (forward primer, F) 5'-AAGGAGACAGCACGGATCCTC-3' (reverse primer, R)	123	NM_004322.3
Akt1	v-akt murine thymoma viral oncogene homolog 1	5'-AGAGAAGCCACGCTGTCCTCT-3' (forward primer, F) 5'-CCGCAGGATAGTTTTCTTCCC-3' (reverse primer, R)	102	NM_001014432.1
AIF	mitochondrial apoptosis inducing factor	5'-AGTAGTTTGCCCACAGTTGGTGTT-3' (forward primer, F) 5'-TCACTCTCTGATCGGATACCAGTTC-3' (reverse primer, R)	101	NM_AL049703.1
Casp 8	caspase 8, apoptosis-related cysteine peptidase	5'-GATATATCCCGGATGAGGCTGAC-3' (forward primer, F) 5'-TGACTGGATGTACCAGGTTCCC-3' (reverse primer, R)	101	NM_001080124.1
Casp 3	caspase 3, apoptosis-related cysteine peptidase	5'-GCCTGAGCAGAGACATGACTCA-3' (forward primer, F) 5'-TCATCCACACATACCAGTGCG-3' (reverse primer, R)	106	NM_004346.3
Casp 9	caspase 9, apoptosis-related cysteine peptidase	5'-TGACTTGTGTCCCATGATCCC-3' (forward primer, F) 5'-AATGTACAGGACAGCCTCACAGC-3' (reverse primer, R)	108	NM_001229.3
ACTB	actin, beta	5'-TCACCGAGCGCGGCT-3' (forward primer, F) 5'-TAATGTCACGCACGATTTCCC-3' (reverse primer, R)	60	NM_001101.3

cDNA samples (@ 20 µl aliquot) were stored at –20°C. For real-time PCR analysis, the cDNAs were amplified in a StepOnePlus Real-Time PCR system (Applied Biosystems, CA, USA) using Fast SYBR Green Master Mix (Applied Biosystems, CA, USA) (Appendix 7). Table 3.1 lists primer sequences used for quantitative real-time RT-PCR analysis. All analyses were performed in triplicate, and the gene expression

levels were normalized by housekeeping gene ACTB. Fold changes in gene expression were calculated based on the standard curve constructed with the StepOne software-calibration.

3.2.6. Western blot analysis

Protein was isolated by using PRO-PREP^{TM} Protein Extraction Solution from 2.5×10^6cell lysates that had been treated with 6 μg/ml arctigenin for 24 h (Appendix 8). The concentration of protein was measured by using Quant-iT^{TM} Protein Assay Kit (Invitrogen, USA) according to the manufacture's procedures (Appendix 9). Protein samples were dissolved in equal amount of sample buffer solution (EzApply, Atta, Osaka, Japan). Proteins (10 μg) were separated at 15-20 mA on 12.5 % SDS-PAGE (e-PAGEL, E-R12.5L, ATTO, Tokyo, Japan), with running buffer (EzRun, ATTO, Tokyo,Japan) . The proteins were transferred to PVDF membranes by using the iBlot^{TM} Dry Blotting System (Appendix 10). The PVDF membranes were treated with the blocking buffer (Blocking One, Nacalai Tesque, Kyoto, Japan) for 1 h and washed with TBS (1xPBS in 0.1% Tween-20). Subsequently, the membranes were incubated with primary antibody (β-actin, CDK2, p-CDK, CDK7, Cyclin E, Cyclin H, Rb, and NPAT) with gentle shaking overnight. After washing, the membranes were incubated with anti-rabbit IgG, HRP-linked antibody as the secondary antibody for 1 h and then washed again. The detection of protein expression targets was performed by luminescent image analyzer (ImageQuant LAS 4000 mini, GE Healthcare, Uppsala, Sweden) using Amersham ECL Advance Western Blotting Detection Kit (GE Healthcare, Buckinghamshire). Relative intensity (%) of protein bands was measured using software of Image Processing and Analysis (ImageJ, Version 1.47d).

3.2 7. Effect of glutathione depletion

Cells suspended in DMEM were seeded at 1×10^4 cells (100 μl) per well of 96-well plates and pre-incubated in humidified atmosphere 5% CO_2 at 37°C for 10 hours. Cells was incubated with 50 μM of glutathione inhibitor-BSO (DL-Buthionine-[S,R]-sulfoximine) for 14 hours, and successively treated with varying concentration of arctigenin for 24 h, and the cell viability was determined by MTS assay kit (Appendix 1). All the experiments were performed in triplicate, and the cell viability was expressed as the relative viability of the sample-treated cell against untreated cell (control).

The ED_{50} values were estimated by fitting the following formula to the full survival curves: $y=\beta_3+ \beta_4/\{1+\exp(\beta_1+\beta_2 x)\}$ where y = cell viability, x = concentration of the test substance in the medium; $\beta_1-\beta_4$=constant. Fitting the formula after transformation to the linear model was done by simple regression analysis program (R Version 2.11.1). ED_{50} of arctigenin treated in the presence of BSO was compared with that of treated without BSO in order to identify the interaction between arctigenin and glutathione status in the various cell lines.

3.2.8. Statistical analysis

The data are expressed as mean ± standard deviation. Statistical significance between pairs of mean was evaluated by Student's *t* test ($p < 0.05$ or $p < 0.01$).

3.3. Results

3.3.1. Specific cytotoxicity of arctigenin in various cell lines

Fig 3.1 shows the effect of arctigenin concentration on cell viability of human lung cancer (A549) and human normal (OUMS-36T-2F) cells. It was noted that human normal cells (i.e. OUMS-36 and OUMS-36T-5F) are resistant while A549 cancer cells and OUMS-36T-2F normal cells were susceptible to arctigenin. A549 cancer cell was found to be most sensitive to arctigenin treatment among the cells studied, reinforcing the cancer specific cytotoxicity of arctigenin.

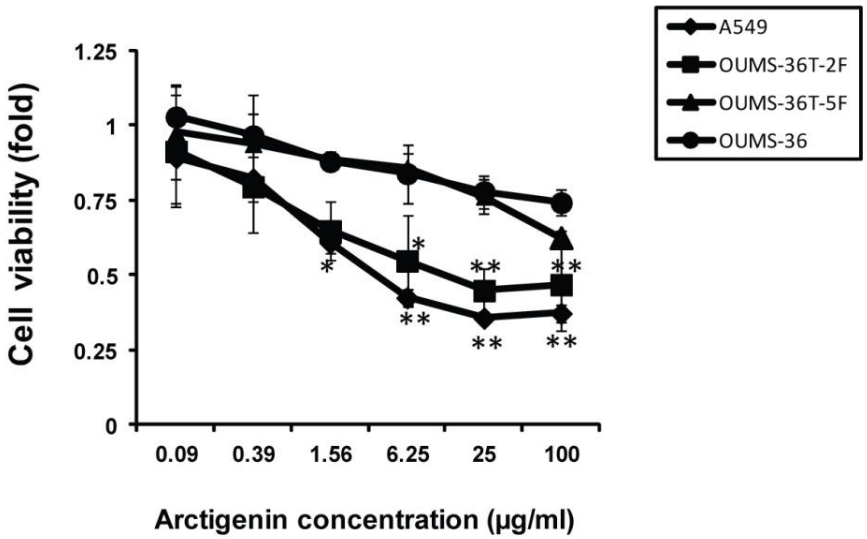


Fig. 3.1 Effect of arctigenin concentration on the viability of various human cell lines. Human lung adenocarcinoma (A549) cells and human normal embryo fibroblast (OUMS-36, OUMS-36T-2F, and OUMS-36T-5F) were treated with indicated concentration of arctigenin for 24 h. Cell viability was expressed as the ratio of treated to untreated cell. Data are mean ± SD of triplicate analysis. *$p<0.05$; **$p<0.01$ versus the untreated cell (control).

3.3.2. Effect of arctigenin on cell cycle of various cell lines.

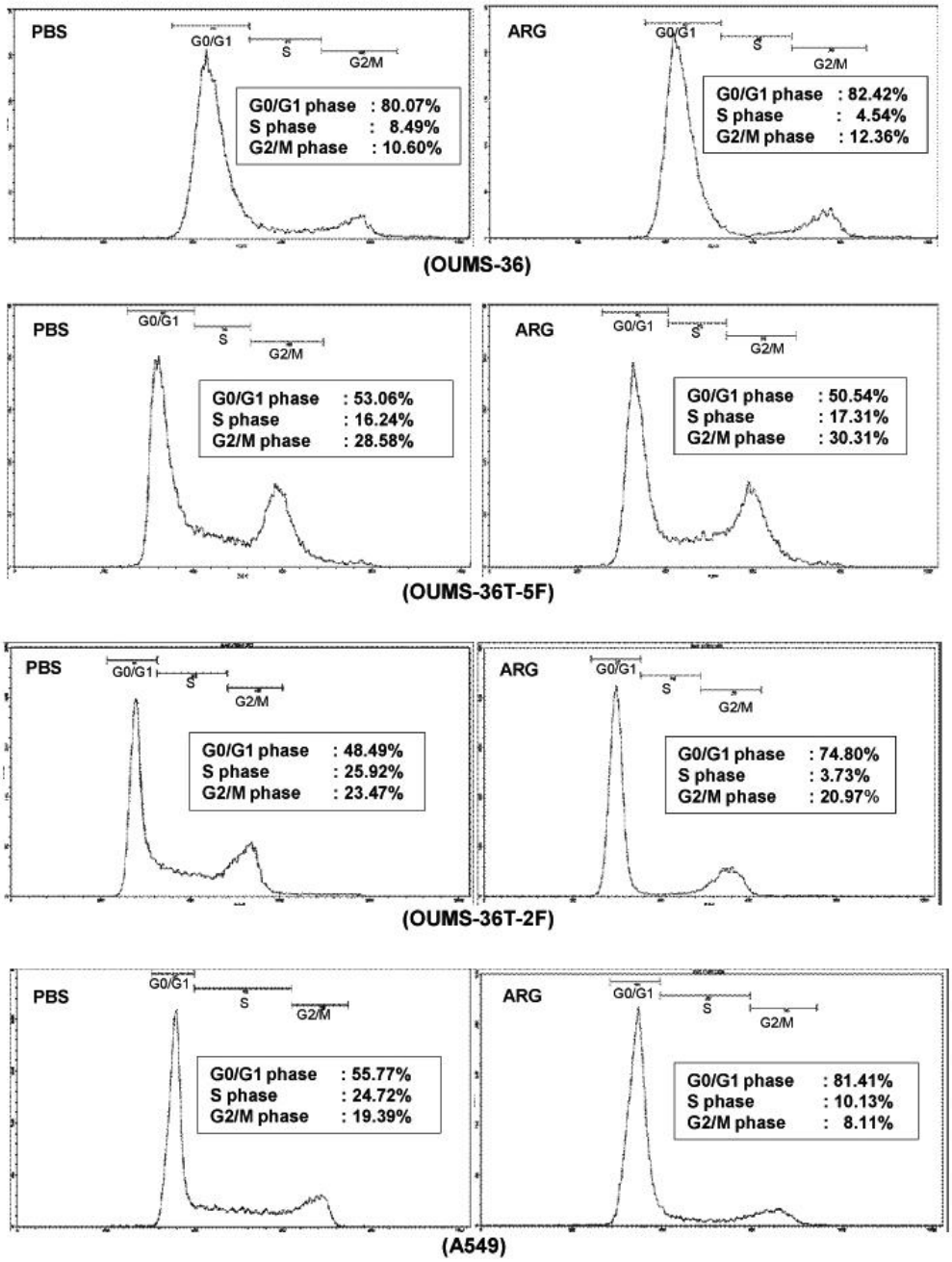


Fig. 3.2 Effect of arctigenin on the cell-cycle of human lung adenocarcinoma (A549) and human normal embryo fibroblast (OUMS-36, OUMS-36T-2F and OUMS-36T-5F) cells. The cell-cycle distribution of cells after treatment of arctigenin (6 μg/mL) or PBS (control) for 24 h was analyzed by flow cytometric histogram analysis. Square legends of each histogram indicate the percentages of cell population in different phases of the cell cycle.

The flow-cytometric histogram demonstrates that the cell population percentage of G0/G1 phase is significantly increased from 55.77% (control) to 81.41% (arctigenin treated cells) in A549, and from 48.49% (control) to 74.80% (arctigenin treated cells) in OUMS-36T-2F normal cell, respectively (Fig. 3.2). This indicated that arctigenin with concentration of 6 μg/mL arrested the cycles of both cells at G_0/G_1 phase. In contrast, there were no significant changes in the cell population of G0/G1 phases for two other normal cells (OUMS-36 and OUMS-36T-5F). These observations suggested that specific cytotoxicity of arctigenin was mainly due to inhibition of cell growth via the cell cycle arrest at G0/G1 phase.

3.3.3. Effect of arctigenin on the expression of apoptosis-related genes

It has been reported that arctigenin induced apoptosis through the caspase-3 activation in A549 lung cancer (Susanti et al., 2012). The current study elaborates more insight into the signaling pathway of apoptosis in arctigenin treated cells. Thus, this study analyzed the expression of caspase-3 and another apoptosis related genes such as pro-apoptosis (Bax, Fas, Bad, AIF, Caspase-8 and Caspase-9) and anti-apoptosis (Akt-1 and Bcl-2) members.

Figure 3.3 shows the effect of arctigenin on the expressions of apoptosis-related genes. Arctigenin treatment up-regulated pro-apoptosis genes inA549 lung cancer, except for Bad gene. Arctigenin also up-regulated anti-apoptosis gene of Bcl-2, and down-regulated another anti-apoptosis gene, Akt-1. In contrast to these observations in A549 cell, arctigenin had no effect on these anti-apoptotic genes, and largely decreased the expression of some pro-apoptotic genes in both normal cells, OUMS-36 and OUMS-36T-5F.

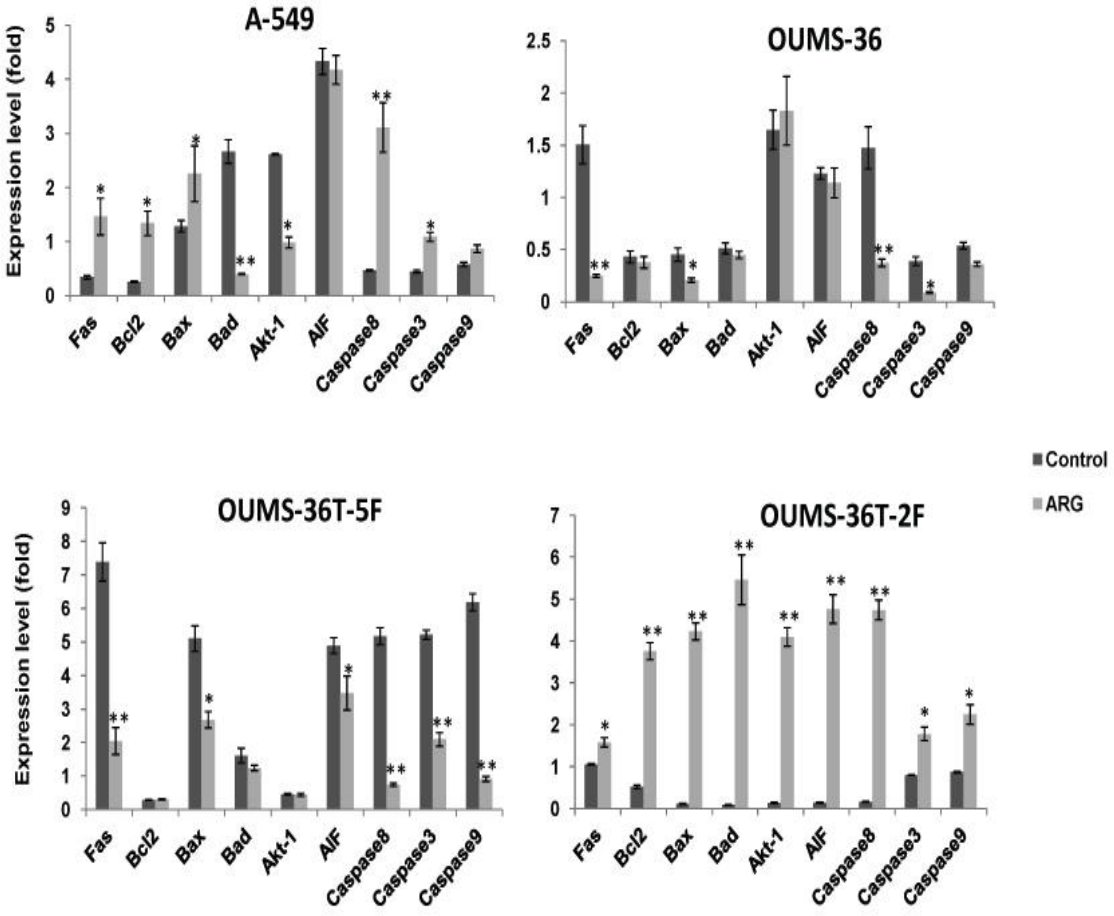


Fig. 3.3 Effect of arctigenin on the expression of apoptosis-related genes in human lung adenocarcinoma (A549) and human normal embryo fibroblast (OUMS-36; OUMS-36T-2F; and OUMS-36T-5F) cells. Gene expression was analyzed by quantitative real time RT-PCR. cDNA was reverse transcribed from total RNA isolated from arctigenin (ARG = 6 µg/mL) and PBS (control) treated cells for 24 h. Description of genes was tabulated in Table 4.1. Value was expressed as the fold change after the expression of each target gene normalized with the house keeping gene. Data are mean ±SD of triplicate analysis. *$p<0.05$; **$p<0.01$ versus the untreated cells (control).

Furthermore, it is noteworthy that arctigenin treatment had no effect on the expression of apoptosis related genes in OUMS-36T-2F (which is a normal cell). Arctigenin treatment significantly up-regulated both pro- and anti-apoptosis genes in this cell. These data suggested that mechanism of action of arctigenin showed variation between types of cells.

3.3.4. Effect of arctigenin on the expression of cell cycle regulatory proteins in various cell lines

Arctigenin has been demonstrated specifically to inhibit the proliferation of cancer cells by suppressing the growth of A549 lung cancer without any adverse effect to normal cells (Susanti et al., 2012). Our study aimed at exploration of molecular mechanism of arctigenin to inhibit cell proliferation. In this section, Western blotting analysis elucidated the effect of arctigenin on some regulatory proteins in cell cycle (such as G0/G1 phase).

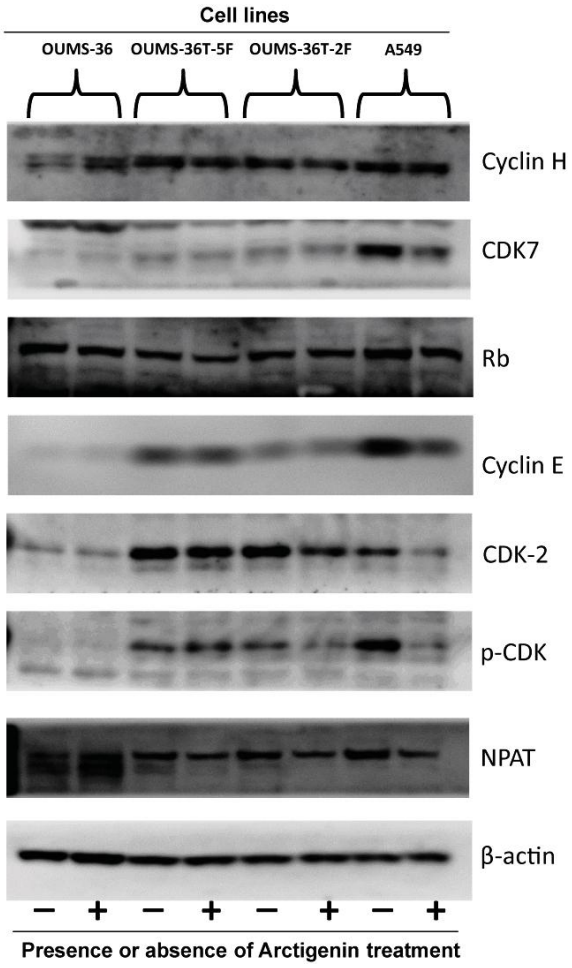


Fig. 3.4 Effect of arctigenin on the expression of cell cycle regulatory protein. Human lung adenocarcinoma (A549) cells and human normal embryo fibroblast (OUMS-36, OUMS-36T-2F and OUMS-36T-5F) were treated with arctigenin (6μg/mL) for 24 h, and 10 μg cell lysates was subjected to SDS-PAGE. Cells without arctigenin were treated with PBS.

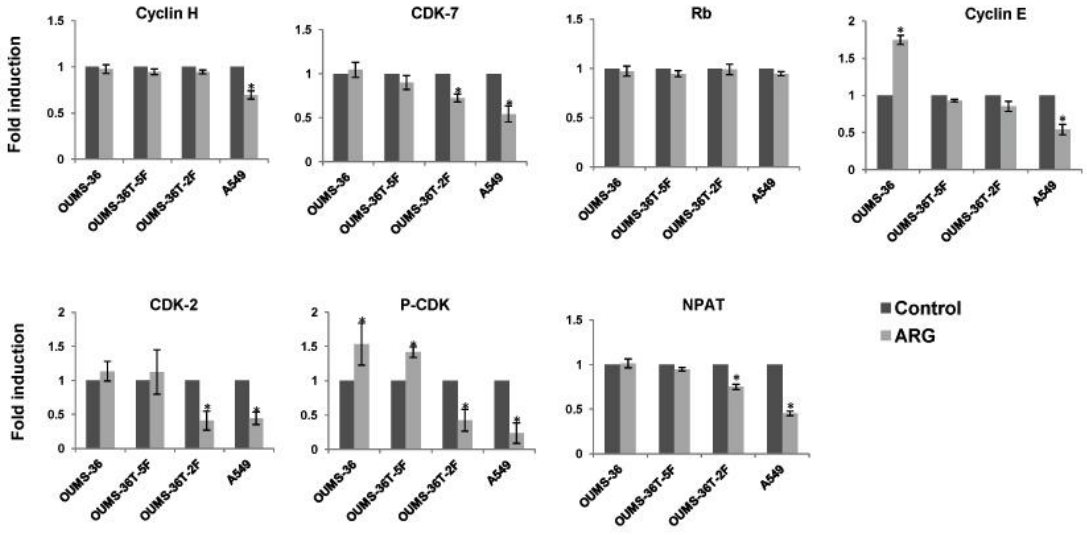


Fig. 3.5 Quantitative expression of the result of Western blotting analaysis shown in Fig. 3.4. Protein band intensity was quantified by ImageJ software, and values were expressed as the ratio of treated to untreated control. Data are mean ±SD (n=3). *p<0.05; **p<0.01 versus the untreated cells (control).

Arctigenin (6 μg/mL) influenced the level of regulatory proteins involved in G_1/S checkpoint signaling such as cyclin H, CDK7, cyclin E, CDK2, p-CDK, and NPAT (Fig. 3.4). Responses to arctigenin again appeared to be associated with the malignancy of the cells, cancer or normal (Fig. 3.5). In A549 lung cancer cells, arctigenin significantly decreased the concetration of cyclin H, CDK7, cyclin E, CDK2, p-CDK, and NPAT protein. Similarly, arctigenin treatment lowered the concentration of these proteins in OUMS-36T-2F normal cells, except for cyclin H. In contrast, no effect was seen with actigenin treatment of other two types of normal cells (i.e. OUMS-36 and OUMS-36T-5F). On the other hand, arctigenin increased the concentration of p-CDK in both OUMS-36 and OUMS-36T-5F normal cell, and cyclin E only in OUMS-36 cells. These data supported our previous observation that arctigenin arrested the cell cycle of cancer cells at G_0/G_1 phase. Furthermore, no significant difference was noted in the concentration of Rb protein between arctigenin treated and untreated cells, suggesting that Rb protein was not involved in cell cycle arrest by arctigenin. Arctigenin may arrest

cell cycle of A549 at G_0/G_1 phase through the reduction of NPAT protein via the down-regulation of either cyclin E/CDK2 or cyclin H/CDK7 complex.

3.3.5. Effect of glutathione depletion on the cytotoxicity of arctigenin

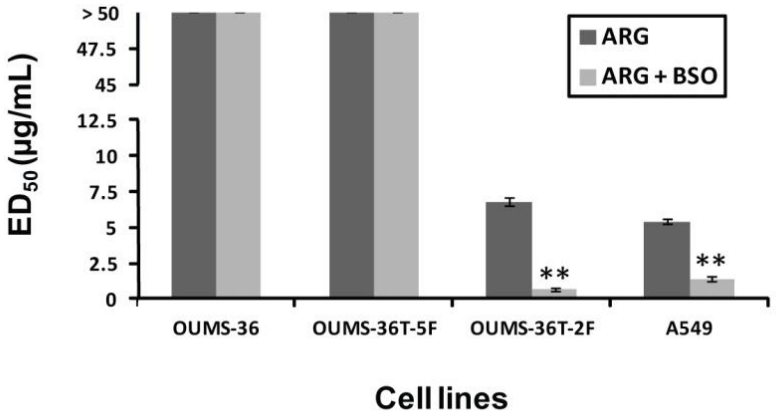


Fig. 3.6 Effect of glutathione depletion on the cytotoxicity of arctigenin. Human lung adenocarcinoma (A549) cells and human normal embryo fibroblast (OUMS-36, OUMS-36T-2F and OUMS-36T-5F) were cultured with 50 µM of BSO-glutathione inhibitor and incubated for 14 h. Cells were incubated with various concentration of arctigenin (ARG) for another 24 h. MTS assay and ED_{50} determination were performed subsequently. Data are mean ±SD (n=3). *$p<0.05$; **$p<0.01$ versus the untreated cells (control).

Our previous study noted that the responses of normal cell lines (OUMS-36, OUMS-36T-5F and OUMS-36T-2F) to arctigenin are not necessarily consistent. OUMS-36 and OUMS-36T-5F are resistant to arctigenin, while OUMS-36T-2F is sensitive and showed almost similar response as A549 lung cancer cell did. Clarification of another mechanism underlying these different responses is utmost important. By using BSO, a glutathione inhibitor agent, we investigate the effect of glutathione depletion on the sensitivity of various cell lines to arctigenin treatment.

ED_{50} of arctigenin was significantly reduced by BSO pretreatment in A549 lung cancer and OUMS-36T-2F normal cells. Whereas, no changes were noted in ED_{50} of arctigenin with OUMS-36 and OUMS-36T-5F normal cells (Fig. 3.6). This suggested that the glutathione content of A549 and OUMS-36T-2F explained, in part, their susceptibility to arctigenin.

3.4. Discussion

We screened 365 KM, and found the occurrence of lung cancer specific cytotoxicity in 9 plant species. Among them, *Arctium lappa* L. contained the cancer specific agent of arctigenin (Susanti et al., 2012), and its selectivity was reinforced in this study as shown in Fig 3.1.

Several mechanism for the cytotoxicity of arctigenin has been reported in previous studies either through the approach of anti-austerity (glucose deprivation cells) or austerity strategy (normal condition cells) (Awale et al., 2006; Sun et al., 2011; Kim et al., 2010; Gu et al., 2012; Yoo et al., 2010; Yao et al., 2011; Jeong et al., 2011). The molecular mechanism for the specific cytotoxicity of arctigenin in lung cancer has been conducted by utilizing austerity strategy approach in which A549 lung cancer cells are starved by glucose deprivation (Gu et al., 2012). It is noteworthy that austerity strategy is the novel concept for cancer therapy based on the idea that cancer cells, especially those of clinically hypo-vascular tumors, might have acquired an ability to tolerate with inadequate blood supply by changing their energy metabolism (Lu et al., 2004; Awale et al., 2012). Anatomically, lung is the organ that is rich in blood vessel because its function as a site for gas exchange of the respiratory system. Through the blood vessel and alveolar sacs, gas exchange between O_2/CO_2 occurs via diffusion process. Based on this anatomical feature, it is quite reasonable to assume that lung cancer is not a group

of hypo-vascular tumor. For this reason, this study elucidates the molecular action of arctigenin in lung cancer with a different approach of normal cell culture conditions.

Apoptosis is an evolutionary process that highly conserved to remove either undesired cells or damaged single cells, resulting in the maintenance of cell number. Cell from various human malignancies has a decreased ability to undergo apoptosis in response to various physiologic stimuli (Jones et al., 1997). For this reason, the induction of apoptosis has become a target in cancer chemoprevention and chemotherapy (Quasney et al., 2001). Our previous study demonstrated that arctigenin specifically inhibited cell proliferation of lung adenocarcinoma, and induced apoptosis via the activation of final apoptosis executor enzyme called caspase-3 without influencing normal cells (Susanti et al., 2012).

In this study, we demonstrated that arctigenin selectively induced apoptosis by up-regulation of pro-apoptosis genes (Bax, Fas, Caspase-3, 8 and 9) and by down-regulation of anti-apoptosis gene (Akt-1) (Fig 3.3). However, Bad (pro-apoptosis) and Bcl-2 (anti-apoptosis) was down- and conversely up-regulated respectively by arctigenin in A549 cancer cells. It has been demonstrated that Bad activity is regulated by phosphorylation via the action of Akt-1. BAD binds with Bcl-2/Bcl-$_{XL}$ in cytosol, and represses their anti-apoptotic activity. Phosphorylation of BAD by Akt-1 promotes its dissociation from the complex, and relocates Bcl-2/Bcl-$_{XL}$ to mitochondria, hence inactivates the apoptotic process (Alberts et al., 2002). Thus, induction of apoptosis by arctigenin may be mediated via the suppression of Akt-1 (Fig. 3.7). However some of the gene expression profile in A549 cancer cells appeared to argue against this explanation: arctigenin increased the expression of anti-apoptotic gene Bcl-2, and decreased apoptotic gene BAD. The role of Akt-1 signaling may have limited significance in the induction of apoptosis by arctigenin. Further studies on the protein

profile are needed to draw the final conclusion on signal transduction by arctigenin to induce apoptosis.

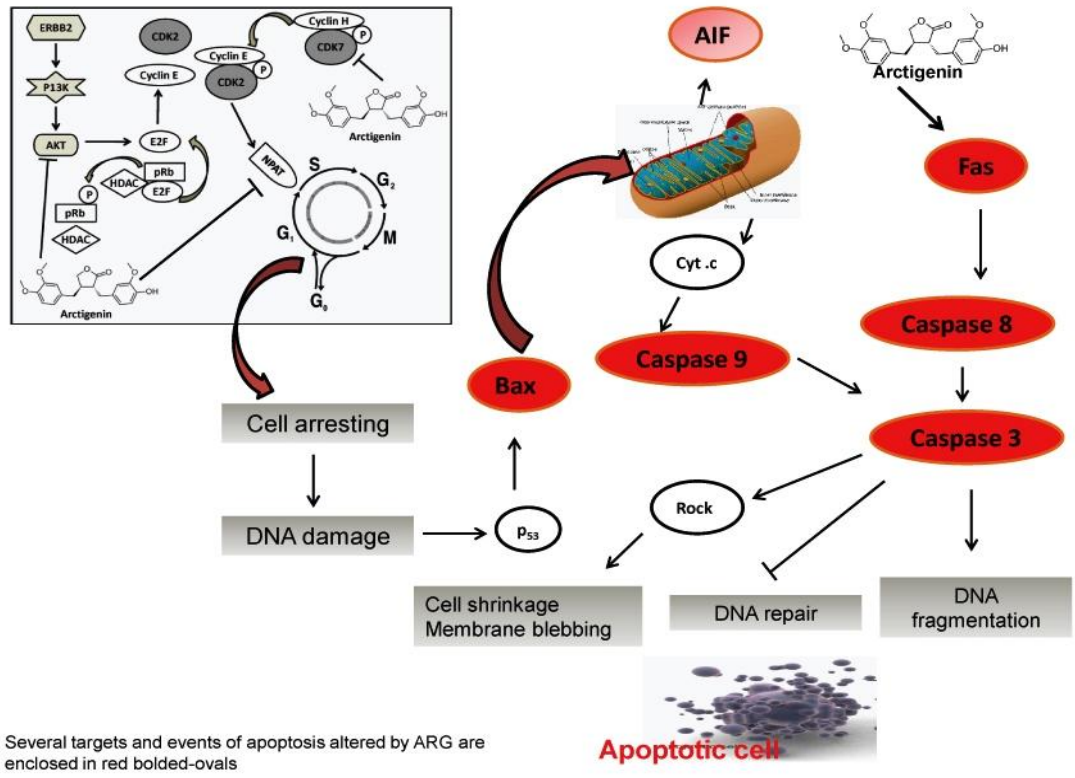


Fig. 3.7 Schematic illustration of arctigenin action to induce apoptosis linked with cell cycle mechanism in A549 (lung adenocarcinoma) cells. Several targets and events of apoptosis altered by arctigenin are enclosed in red bolded-ovals.

In addition to the role in apoptosis induction, Akt is known to play a role in the control of cell cycle (Fig. 3.8). Under various circumstances, activation of Akt overcomes the cell cycle arrest at G_1 phase, thus enable proliferation and survival of cells (Ramaswamy et al., 1999). It has also been shown that inhibition of PI3K/Akt signaling arrested cell cycle at G1 phase and induced apoptosis in Hodgkin lymphoma (Georgakis e al., 2005). Similarly, suppression of Akt-1 in ERBB-2/Akt signaling pathway inhibited E2F-1(a protein with a crucial role in controlling cell cycle

progression), and induced G_0/G_1 cell cycle arrest (Guo et al., 2010). The decreased expression of Akt-1 by arctigenin thus may contribute in part to the cell cycle arrest at G_0/G_1 phase in this study (Fig. 3.2).

The cell cycle arrest and apoptosis are closely linked in mammal cells (Trivedi et al., 2005). The sensitivity of cell to certain apoptosis often depends on the cell cycle, and inhibition of the cell cycle has been considered as a target for the management of cancer (Sherr, 1994; Kornberg, 1999). The cell cycle progression is mediated by multiple phosphorylations mediated sequentially by a series of cyclin/CDK complex, and G_1-phase progression is mediated by combined activity of cyclin D_1/CDK-4 and cyclin E/CDK-2 complexes (Hume et al., 2008) that target the retinoblastoma (Rb) tumor suppressor protein. It has been an established view that the Rb protein plays a crucial role in the control of cell cycle. Rb can actively inhibit cell cycle progression when it is dephosphorylated, while this function is lost when Rb is phosphorylated (Vietri et al., 2006). Rb protein functions primarily to prevent unscheduled entry into the mitotic cell cycle through the ability to mediate the transcriptional repression of genes required for DNA replication and mitosis. Rb activity is engaged to inhibit the transcription of multiple genes required for S-phase entry, and to arrest cell cycle at G_0/G_1 phase. Thus, it has been shown that perturbations of the Rb pathway confer a synonymous proliferative advantage to tumor cells. Mitogens therefore often counteract the action of Rb through signals that promote activation of cyclin-CDK complex, which phosphorylate Rb and attenuate its capacity to inhibit transcription required for continued cell proliferation. Phosphorylation of Rb disrupts complexes with E2Fs, and allows the cells to enter S-phase (Buchkovich et al., 1989; Kato et al., 1993; Brehm et al., 1998).

Arctigenin in this study increased the G_1 populations of cell cycle in A549 cancer cells, and induced G_1/S arrest. Investigations on cell cycle regulators found that arctigenin decreased the level of cyclin H, CDK-7, cyclin E, CDK-2, P-CDK and NPAT protein, and had no effect on Rb protein (Fig. 3.5). Previous study has shown that arctigenin arrested the cell cycle at G_0/G_1 phase thorough the phosphorylation state of Rb protein probably via the down-regulation of cyclin E/CDK2 or cyclin D/CDK4 in human gastric cancer (Jeong et al., 2011). It is also demonstrated that arctigenin arrested the cell cycle of human colorectal cancer cells in G_2/M phase by modulation of Wnt/β-catenin signaling pathway (Yoo et al., 2010). These observations collectively point the variation in the cell cycle arrest point and the signaling pathway in cancer cells treated with arctigenin. Our present study observed cell cycle arrest of A549 cells in G_0/G_1 phase and suppression of cyclin E/CDK-2 level suggesting that similar mechanism operating in gastric cancer cells involved in the growth inhibition of lung cancer cells. Furthermore, it is noteworthy that arctigenin decreased the level of cyclin H/CDK-7. In addition to cyclin binding, CDK activity is also regulated by phosphorylation on conserved threonine and tyrosine residues. Thus, full activation of CDK-2 requires phosphorylation of threonine 160 brought by cyclin H/CDK-7 complex, also called CDK activating kinase (CAK). Our observation suggested that arctigenin affected the all cycle phases through down-regulation of cyclin H/CDK-7 complex for the first time (Fig. 3.8).

Modulation of signaling pathway by arctigenin appeared to be converged into the regulation of the final effecter proteins called NPAT to control the cell proliferation process. NPAT is required for the transcriptional activation of histone genes. The bulk of histone proteins are assembled with genomic DNA into chromosomes, and the biosynthesis of histones is tightly coordinated and coupled with DNA synthesis during S

phase of cell cycle. Perturbation of the coordination and coupling causes the loss of chromosomes, DNA damage, and results in cell cycle arrest. Histone biosynthesis is regulated at both transcriptional and posttranscriptional levels, and the suppression of NPAT expression impedes expression of all histone subtypes (Zhao et al., 2000). It has been demonstrated that the phosphorylation of NPAT by cyclin E/CDK-2 associates with histone gene promoter. Therefore, it is likely that inhibition of cyclin E/CDK2 led to down-regulated NPAT in this study (Fig. 3.4 and 3.5).

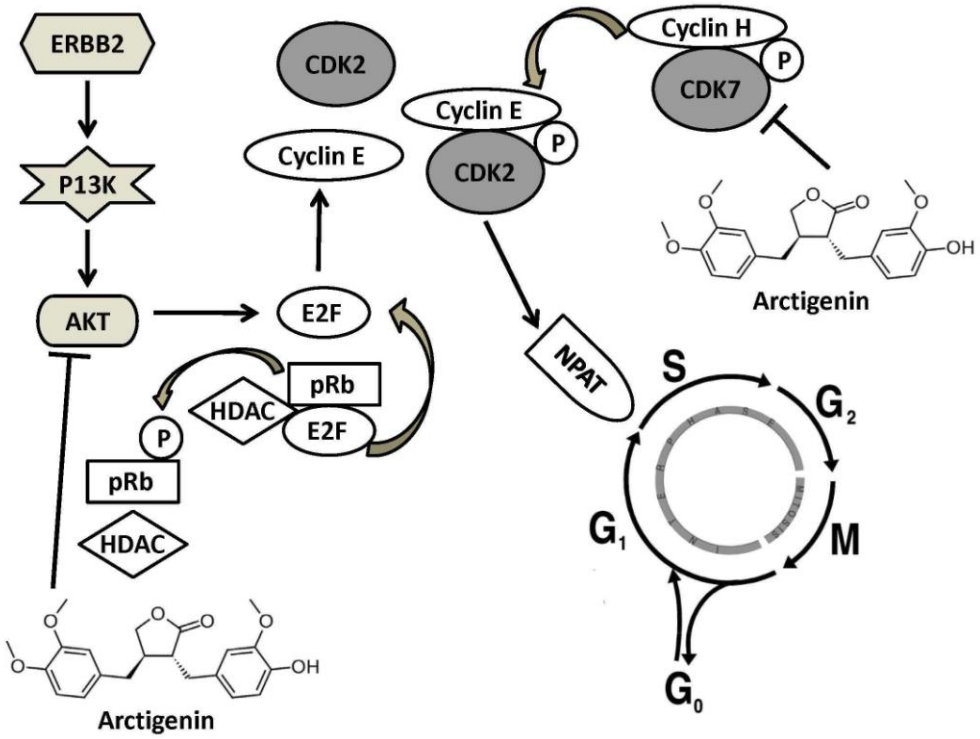


Fig. 3.8 Schematic illustration about proposed mechanism of arctigenin action induces G_0/G_1 phase arrest of the A549 (lung adenocarcinoma) cell cycles.

Previous study reported that the cytotoxicity of *Arctium lappa* L. extract is reduced by intracellular GSH (Moritani et al., 1996). Inclusion of GSH synthase inhibitor enhanced the sensitivity of A549 and OUMS-36-2F to arctigenin (Fig. 3.6) This finding suggested that GSH content of cancer cell also a factor to determine their

susceptibility to arctigenin. Thus GSH content, as well as control of cell cycle, may become of a new target for development of anticancer agent.

Our and other previous studies suggested that anticancer activity of arctigenin is fairly cell-specific. The cytotoxicity of arctigenin is only specific to the cancer cell, while the normal cell is resistant to this cytotoxicity. However, this study also disclosed that the cytotoxicity toward normal cells showed variation between cell types. OUMS-3T-2F normal cell is sensitive to arctigenin, as was the case for A549 cancer cell, while OUMS-36 and OUMS-36T-5F are resistant to this agent. Both OUMS-3T-2F and OUMS-3T-5F were produced by transformation of OUMS-36 primary cells with hTRT gene (human telomerase reverse transcriptase gene in pMX) (Kouchi and Namba, 2000). RFLP analysis (short tandem repeat (STR)-PCR) of genome DNA found that OUMS-3T-2F only differs from other OUMS-36 series in the length of microsatellite markers vWA: the STR 20 for OUMS-3T-2F was replaced with STR21 in OUMS-3T-5F. The difference in the genome DNA sequence close to this marker was obviously responsible for the susceptibility to arctigenin, and clarification of this point promisingly shed the light on the mechanism for the cancer specific cytotoxicity of arctigenin. Continuous work along this line will contribute to the development of a novel anticancer agent of low side effect.

3.5. Conclusion

The mechanism of action of arctigenin leading to specific cytotoxicity was studied. Arctigenin selectively arrested the cell cycle of cancer cells at G_0/G_1 phase, while induced apoptosis via modulation of gene expression in Akt-1 related signaling pathway. Furthermore, the cell cycle arrest in cancer cells at G_0/G_1 phase was found to be effected by the down-regulation of NPAT protein via the suppression of either cyclin

E/CDK2 or cyclin H/CDK7. Furthermore, GSH synthase inhibitor specifically enhanced the cytotoxicity of arctigenin, suggesting that intracellular GSH content appeared to be another factor to influence the susceptibility of cancer cells to arctigenin.

Chapter 4

Studies on the enhancement of cancer-selective cytotoxicity of Kampo medicine by combination

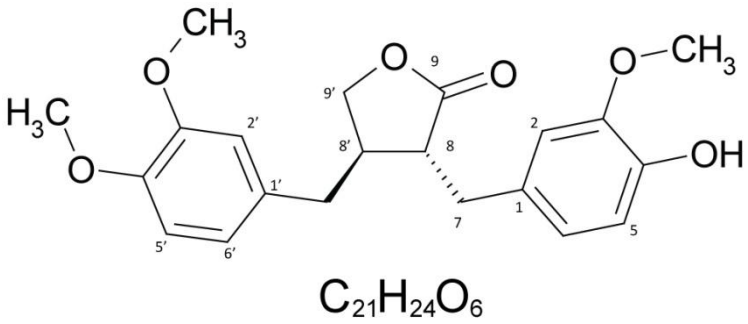
4.1. Introduction

Kampo medicine (KM) has its origin in traditional medicine in China from where it, spread to Japan in the fifth and sixth centuries (Alphen et al., 1995). Herbal plants in the KM system have been used in Japanese folk medicine ever since. Recently, there has been increasing interest in the use of KM as a complementary/alternative medicine for a variety of pathological conditions (Tamayo et al., 2000). It has been reported that KM alleviated inflammatory reactions in some pathogenic states, and effectively ameliorated the side effects of cancer chemotherapy, thereby improving the quality of life (Ieong et al., 2006; Mukherjee et al., 2007; Sinclair et al., 1998; Kim et al., 2007; Kamaleeswari, et al., 2006; Pellati et al., 2002; Murakami et al., 2000).

Cancer has been the world's leading killer disease (Pisani et al., 1999; Greenlee et al., 2001). Various treatment strategies have been employed, such as chemotherapy, surgery, radiation, and immunotherapy using monoclonal antibodies (Skeel, 2003). Although chemotherapy is the most common choice for cancer treatment, its drawbacks are the severe side effects (Camp-Sorrell et al., 2000). These reduce the quality of life of cancer patients, and they have been a major issue in cancer treatment (Cline, 1984; de Jong, 2002).

We have been working on the isolation of anti-cancer agents with low side effects from medicinal or herbal plants (Iwasaki et al., 2006; Oku et al., 2007; Iwasaki et al., 2009; Susanti et al., 2012). KM has been used to promote physical rehabilitation and to reduce the adverse side effects after chemotherapy or radiation therapy, leading to

improved life quality for cancer patients (Yamada, 2006). Based on KM's clinical history, it is quite reasonable to postulate that these herbal plants contain natural cancer-specific agents with low side effects. Thus, in our previous study, we isolated, and identified arctigenin (Fig. 4.1) as an active component of cancer selective cytotoxicity from the medicinal herb *Arctium lappa.* L. (Susanti et al., 2012). Arctigenin specifically inhibited the growth of lung cancer cells A549, and induced apoptosis with no adverse effect on normal cells (Susanti et al., 2012).



$C_{21}H_{24}O_6$

Fig. 4.1 The chemical structure of arctigenin identified as the cancer-selective cytotoxic component of the extract from *Arctium lappa* L.

KM represents a mixture of biologically active compounds, and therefore the effect may rely on interactions between its individual components. Although KM appears to have various advantages over western drugs in a wide array of clinical applications, it should also be taken into consideration that KM components may have antagonistic or synergistic interactions with each other as in any mixed medication systems (Watanabe, 2010). Empirical observations of traditional practices prove that some herbal plant extracts applied as a composite formula provided an effective cure (Wenjing et al., 2006). However, the scientific evidence of its efficacy and safety is limited because of the lack of experimental and clinical studies. Therefore, in order to maximize the cancer-specific toxicity, and minimize the side effects of KM, it is

important to evaluate its efficacy invitro as single dose or as composite formulation prior to clinical trials (Buchanan, et al., 2005).

The aim of this study was therefore to test the efficacy of the combinations of herbal extracts for their cancer selectivity, and to screen preliminarily the best extract coupled with arctigenin to enhance cancer-specific cytotoxicity. The target cancer cell in this study was lung adenocarcinoma. Lung cancer one of the world's leading killer diseases and the result of treatment with conventional chemotherapeutics have been disappointing. Further investigations are therefore urgently needed to combat this disease.

4.2. **Materials and methods**

4.2.1. Preparation of plant extracts

We used 9 herbal plants from the 364 used in our previous study, with selection based on their specific toxicity to A549 lung cancer cells. Those selected were as follows: *Citrus unshiu* (pericarp), *Arctium lappa* (seeds), *Citrus aurantium* (fruit), *Citrus reticulata* (unripe fruit), *Prunus mume* (unripe fruit), *Acorus calamus* (root), *Ligusticum wallichii* (rhizome), *Phyllostachys nigra* (leaf), and *Carum carvi* (seeds). Air-dried whole plants were purchased from Kojima Kampo, Osaka, Japan. Plant samples (0.5 gram) frozen in liquid nitrogen were powdered, and then extracted with 10 ml of 50% methanol (MeOH) at room temperature for 48 h. The extracts were filtered through a sterile filter (Millex-LG <0.20 μm) and stored at 6^{o}C in a refrigerator. They represent 100% extract concentration.

4.2.2. Cell culture

The various normal or cancerous human cell lines used for the antitumor assay are listed below. They were purchased from the Japan Cancer Research Resources Bank (JCRB, Ibaragi, Japan). Normal cell lines: WI-38, normal lung diploid fibroblast; KMST-6, human fibroblast; OUMS-36, normal human embryo fibroblast. Cancer cell lines: A549, lung adenocarcinoma; Hep-G2, liver cancer; A431, epidermoid carcinoma. Cells and sub-culture were maintained according to the suppliers recommendations. The culture medium were DMEM and EMEM supplemented with 10% FBS (Fetal Bovine Serum). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37^{o}C.

4.2.3. Cytotoxicity test

Cell viability was measured using the MTS assay kit according to the manufacturer's instructions (Appendix 1). Briefly, the sample or vehicle was added to the 96-well plate, and dried aseptically for 30 min. Cytotoxicity titration curves were constructed with serial dilutions of samples in 96-well microplate. Cells suspended in the appropriate medium and seeded at 1 x 10^3 cells/100μl were pre-cultured overnight, and treated with a single 4% dose of herbal extract for another 24 h. The ratio of cell viability after the treatment to that of an untreated control was taken as cytotoxicity in this study.

4.2.4. Combination test of crude extract

Firstly, the cytotoxicity of the 9 herbal extracts was assessed by using a single treatment on various cancer and normal cell lines. The primary extract from each herbal plant was added to the culture to a final extract concentration of 4% in the medium. The percentage (%) in this case indicates the concentration of the original crude extract in

the culture medium based on the mixing ratio. Subsequently, the effects of combinations of these extracts on the growth of cancer cells were studied using final concentration of 1% for each extract. Cell lines used were: A549, Hep-G2, A431, WI-38, OUMS-36, and KMST-6. For this combination treatment, equal volumes of the original extracts (100%) were mixed, concentrated to dryness, and re-dissolved in 50% MeOH prior to addition to the culture. Thus, a 1 % level of the mixture in the culture medium gives a final MeOH concentration of 0.5% in the medium. Preliminary experiments revealed that MeOH up to a 0.5 % level in the culture medium had no effect on cell viability. Cells seeded at a density of 1,000/well were pre-cultured for 24 h, and then treated with a 1 % dose of the crude extract mixture or PBS (for the control groups) for another 24 h. Viabilities were determined by the MTS assay and expressed as the ratio of the treated to control cells (PBS).

4.2.5. Combination test of arctigenin and crude extract

To test the interactions of arctigenin with other extracts, cytotoxicity assays of crude extracts were conducted in the presence or absence of arctigenin (The, ED_{50} of arctigenin against A549 cells is 5.4 μg/ml). Cells seeded at a density of 1,000/well were pre-cultured for 24 h, and then treated with serial dilutions of one of the 9 herbal extracts (+ or – arctigenin) for another 24 h. The viabilities were determined by MTS assay and expressed as the viability ratio of the treated to untreated cells.

4.2.6. Estimation of ED_{50}

ED_{50} (half-effective dose) was defined as the dose required to reduce cell viability by 50% after the specified test duration. This was determined by fitting the following formula to the titration curve: $y = \beta_3 + \beta_4/\{1+\exp(\beta_1+\beta_2 x)\}$ where y = cell

viability, x = concentration of the test substance in the medium; β_1–β_4 are =constants. Fitting the formula after transformation to a linear model was done by a simple regression analysis program provided by R. Version 2.11.1 (Copyright (C) 2010, The R Foundation for Statistical Computing). The ED_{50} values of crude extract in the presence or absence of arctigenin were compared to discover if there were interactions between arctigenin and the crude herbal plant extract.

4.2.7. Statistical analysis

The data were expressed as mean ± standard deviation. The statistical significance of the differences in the mean values was evaluated by Student's *t*-test. P values less than 0.05 or 0.01 were considered as statistically significant.

4.3. Results

4.3.1. Tumor specific cytotoxicity of crude extract

We evaluated the selective cytotoxicity of a single 4% dose of each plant extract by using various cancer and normal cell lines. Cancer cell lines: lung adenocarcinoma, A549; liver cancer, HepG2; and epidermoid carcinoma, A431. Normal cell lines: lung normal diploid fibroblast, WI-38; human fibroblast, KMST-6; and normal human embryo fibroblast, OUMS-36. Fig. 4. 2 shows that cancer cell lines, as a whole, were more susceptible to these extracts than were the normal cell lines. Of the 9 extracts, that from *A. lappa.L* was found to have the most potent cytotoxicity to the cancer cell lines (A431, A549 and Hep-G2, respectively) with no adverse effect on the normal cell lines. These observations indicate that *A. lappa. L.* extract has anticancer activity with low or

no side effects. Of the cancer cells tested, A549 was the most sensitive to all extracts except for that from *A. lappa. L.*

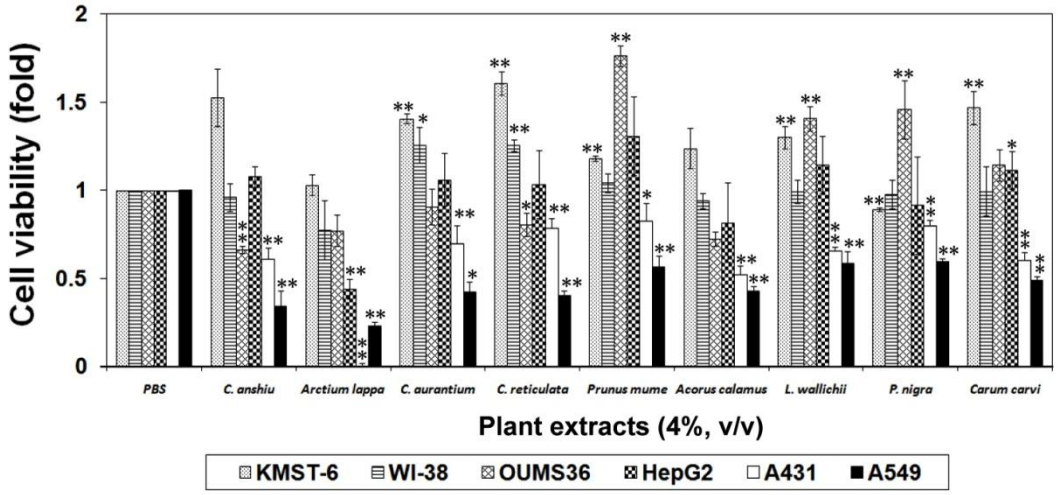


Fig. 4.2 Viabilities of various cancer cells after exposure to 4% dose of 9 herbal extracts. The normal cells used were KMST-6, WI-38, and OUMS-36T-5F. The cancer cells used were HepG2, A431, and A549. For the descriptions of these cells, refer to the Materials and Methods section. Cells seeded at a density of 1,000/well were pre-cultured for 24 h, and treated with a single 4% dose of herbal extract for another 24 h. The viabilities were determined by the MTS assay and expressed as the viability ratio of treated to untreated cells. Data are the mean ± SE of triplicate analyses. *$p < 0.05$; **$p < 0.01$ versus control (PBS).

4.3.2. Combination of crude mixtures

We now examined the effects of combinations of the 9 extracts on the viability of cancer and normal cell lines. As described in the Materials and Methods section, the mixture consisted of equal volumes of the 9 different herbal plant extracts, concentrated to dryness and re-dissolved in 1 part of 50% MeOH so that the concentration of each extract in this mixture was equal to that of original extract. As was the case for a dose of a single herbal plant extract, the viability of cancer cells decreased following the combination treatment. In contrast, normal cells were largely resistant to the mixture at a 1% concentration. Of the cancer cells, A549 was the most susceptible, A431 intermediate, and HepG2 only slightly susceptible to the 1% mixture of these extracts

(Fig. 4.3). It was notable that the medium containing 1% of all 9 herbal extracts completely and selectively suppressed the growth of lung cancer cells (A549) with no any cytotoxicity to normal cells.

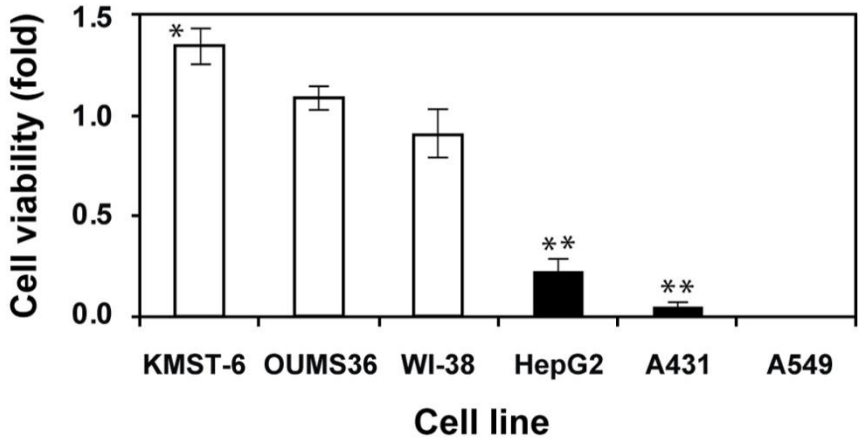


Fig. 4.3 Viabilities of cancer and normal cells after exposure to the composite formula of 9 herbal plant extracts. The normal cells used were WI-38, OUMS-36T-5F, and KMST-6. The cancer cells used were A549, HepG2, and A431. For a more detailed description of these cells, refer to the Materials and Methods section. Cells seeded at a density of 1,000/well were pre-cultured for 24 h, and treated with a 1% dose of the crude extract mixture or PBS (control groups) for another 24 h. The viabilities were determined by the MTS assay and expressed as the viability ratio of the treated to control cells (PBS). Data are the mean ± SE of triplicate analyses. *$p < 0.05$; **$p < 0.01$ versus control (PBS).

4.3.3. Combination of arctigenin with crude extract

Fig. 4.4 shows the cytotoxic effects of 8 extracts on the viability of normal (KMST-6) and cancer (A-549) cells. All extracts showed higher toxicity toward cancer cells than to normal cells, with more pronounced selectivity at higher extract concentrations. Selective cytotoxicity to cancer cells was most clearly manifested by the extracts of *Citrus unshiu* and *Citrus reticulata*. These observations are largely consistent with our previous data (Susanti, 2012), and confirm the specific cytotoxicity to A549 cancer cells of these crude extracts. We isolated the active component from *A.*

lappa. L. and identified it as arctigenin (Fig. 4.1). Arctigenin specifically lowered the viability of cancer cells with no adverse effect on the normal cells (Susanti, 2012). The presence of arctigenin is the reason for the selective cytotoxicity of the extract from *A. lappa.* L., and it may interact synergistically or antagonistically with active components in the crude extracts of other herbal plants. It is therefore important to study these interactions invitro.

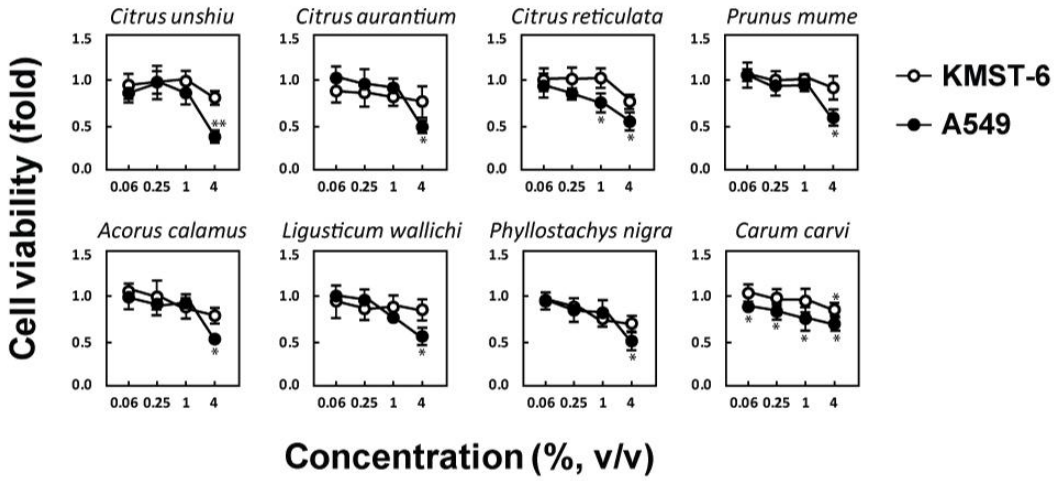


Fig. 4.4 Viabilities of A549 (cancer) and KMTS-6 (normal) cells after exposure to the various concentrations of herbal plant extracts. Open and closed circles show viabilities for normal and cancer cells, respectively. Cells seeded at a density of 1,000/well were pre-cultured for 24 h, and treated with serially diluted 9 herbal extract for another 24 h. The viabilities were determined by the MTS assay and expressed as the viability ratio of the treated to untreated cells. Data are the mean ± SE of triplicate analyses. *$p < 0.05$; **$p < 0.01$ versus the KMST-6 cells.

To examine the interactions between arctigenin and the other crude extracts, a cytotoxicity assay was performed in the presence or absence of arctigenin. We can expect the ED_{50} value of the herbal extracts to decrease if their interaction with arctigenin is synergistic or additive, and conversely, to increase in the case of antagonism. Table 4.1 lists the ED_{50}s of extracts measured in the presence or absence of arctigenin with cancer and normal cells.

Arctigenin caused 3 patterns of changes in the ED_{50} of extracts against cancer cells: decrease, no change, and increase. The ED_{50} values for *Prunus mume*, *Carum carvi*, *Citrus reticulate*, and *Citrus aurantium* against A549 cancer cells were decreased by the presence of arctigenin. For extracts from *Prunus mume* and *Carum carvi* the ED_{50} value for KMST-6 normal cells was not affected by the presence of arctigenin, whereas it decreased with those from *Citrus reticulata* and *Citrus aurantium*.

Table. 4.1 ED_{50} (%) of extracts against A549 and KMST-6 cells in the presence (+) or absence (-) of arctigenin

Plant extract	A549*		KMST-6**	
	-	+	-	+
Prunus mume	**6.5**	**1.89**	**>20**	**>20**
Carum carvi	**>20**	**4.7**	**>20**	**>20**
Citrus reticulata	13	0.8	>20	4
Citrus aurantium	4.7	0.8	>20	6.4
Phyllostachys nigra	5.8	6.2	>20	0.7
Citrus unshiu	2.3	3	>20	> 20
Ligusticum wallichii	6	16.4	>20	> 20
Acorus calamus	4.5	> 20	10.4	6.7

ED_{50} is expressed as % of the concentration of the original extract present in the culture medium, as mentioned in the Materials and Methods section.
*Lung adenocarcinoma cell
**Normal human fibroblast cell

Addition of arctigenin had no effect on ED_{50}s against cancer cells of the extracts from *Phyllostachys nigra* and *Citrus unshiu*, and increased those for *Ligusticum wallichii* and *Acorus calamus.*

4.4. Discussion

The present study confirmed the cancer-selective cytotoxicity of 9 herbal plant extracts chosen from 364 specimens screened in our previous study (Susanti, et al.,

2012), and further examined the enhancement of the cancer selective cytotoxicity by combinations of these extracts.

A mixture containing several different active components (composite formula) usually has greater efficacy than a single ingredients likely due to the synergistic interactions of the ingredients (Wenjing et al., 2006; Xue and Roy, 2003). This view holds true for the current case where combinations of 9 herbal plant extracts were tested. When the 9 extracts were applied individually at a 4% dose, the viability of cancer cells was significantly decreased, but not to zero level, and there was slight toxicity to normal cells in some cases (Fig. 4.2). The combination of all 9 herbal extracts, each at 1 % concentration, however, completely and selectively suppressed the growth of cancer cells with no detrimental cytotoxicity to normal cells (Fig. 4.3). This finding suggests that a single herbal extract, even an effective one, may damage normal cells in high-doses, while a combination of low-doses of active extracts may be very effective in the selective suppression of cancer cell growth. Adverse side effects of the herbal extracts thus might be offset by the interactions between cells and diverse active constituents when extracts are combined (Huang et al., 2004; Ikezoe et al., 2003; Plouzek et al., 1999). Furthermore, combining the active extracts appears to potentiate selectivity toward cancer cells even with low-doses of individual extracts in the mixture. This suggests there may be synergistic effects between diverse components in the mixture of herbal extracts, which may need further investigation in the future. It is likely that combining the extracts brought together the advantages of each type for an overall enhancement of cancer treatment (Wenjing et al., 2006).

Purification of the selective cytotoxic activity in the extract of *A. lappa*. L. resulted in the identification of arctigenin as the active component (Fig. 4.1). Arctigenin is a phenylpropanoid dibenzylbutyrolactone lignan with antioxidant, anti-inflammatory

and antitumor activities (Awale et al., 2006; Cho et al., 2004; Tamayo et al., 2000; Zhao et al., 2009). Arctigenin sepecifically inhibited the proliferation of cancer cells with no detrimental effect on normal cells, and was therefore responsible for the selective cytotoxicity of the extract from *A. lappa*. L. In this context, it is noteworthy that the combination of herbal extracts was very effective in selectively suppressing the growth of cancer cells, suggesting that the combination of active components in the herbal extract may give rise to a new and potent anti-cancer formula. This study therefore aimed to screen for active components that potentiate cancer specific cytotoxicity synergistically or additively in combination with arctigenin. Therefore, a cytotoxicity assay of 8 crude extracts was conducted in the presence or absence of arctigenin (Table 4.1). We can expect the ED_{50} value of the herbal extracts to decrease if their interaction with arctigenin is additive or synergistic, and conversely, to increase in the case of antagonism.

Among the extracts, those of *Prunus mume*, *Carum carvi*, *Citrus reticulate,* and *Citrus aurantium* showed a decreased ED_{50} in the presence of arctigenin, suggesting that arctigenin increased the cytotoxicity of these extracts additively or synergistically. However it is important that the combination should not show increased toxicity to normal cells. From this point of view, combining arctigenin with extracts from *Prunus mume* or *Carum carvi* is preferable because the ED_{50} to cancer cells decreased while normal cells were unaffected. This suggested that the combination potentiated the cancer-specific toxicity with no detrimental effects on normal cells. This was not true for all extracts, as shown when extracts from *Citrus reticulata* or *Citrus aurantium* were combined with arctigenin. The mixtures still manifested selective cytotoxicity to cancer cells, but cytotoxicity to normal cells also increased well as to cancer cells. This may be

due to an overdose of cytotoxic compounds by the combination with arctigenin, and further optimization may be necessary to minimize the cytotoxicity to normal cells.

As shown in Fig. 4.2, the 9 herbal plant extracts applied individually at a 4% dose significantly decreased the viability of cancer cells with slight toxicity to normal cells in some cases. This finding suggests that an over-dose of herbal medicine, even an effective one, might damage normal cells. This might be circumvented by interactions between the wide arrays of compounds in the mixed formulae of Kampo medicine. Both the possible synergies and the reduction in adverse side effect definitely merit further investigation. Optimization with respect to the specific cytotoxicity to cancer cells while ameliorating the toxicity to normal cells might be achieved by appropriate combinations of Kampo medicines. Approaches based on interactions between Kampo-derived individual compounds or herbal plant extracts are necessary to find the optimum Kampo formulation for cancer treatment, and may open a new avenue for cancer chemotherapy.

4.5. Conclusion

These observations suggest that combination of Kampo medicine can optimize cytotoxicity specifically to cancer cells while normal cells experience minimal toxicity. This study thus describes the optimization of Kampo formulations through interactions between Kampo-derived individual compounds or herbal plant extracts, and discusses the importance of this approach to enhance cancer-specific cytotoxicity and to open a new avenue for cancer chemotherapy.

Chapter 5

General Conclusion

Chemotherapy as one of therapeutic options for cancer, until recently still has the drawbacks of severe side effects and dose-limiting toxicity. This study was conducted to provide the scientific evidences for the development of selective anticancer agent by employing Kampo medicine.

We first evaluated the cytotoxicity profiles of 364 herbal plant extracts, using various cancer and normal cell lines. The screening found occurrence of A-549 (human lung adenocarcinoma) specific cytotoxicity in 9 species of herbal plants, especially in the extract of *Arctium lappa* L. Purification of the selective cytotoxicity in the extract of *Arctium lappa* L. resulted in the identification of arctigenin as tumor specific agent. Arctigenin specifically inhibited the proliferation of cancer cells, which might consequently lead to the induction of apoptosis. Thus, this study found that arctigenin was one of the cancer specific phytochemicals, and responsible for the tumor selective cytotoxicity of the herbal medicine.

Next, the mechanism of action of arctigenin leading to specific cytotoxicity was studied. Arctigenin selectively arrested the cell cycle of cancer cells at G_0/G_1 phase through modulation of cell cycle regulatory protein, while induced apoptosis via modulation of gene expression in Akt-1 related signaling pathway. Furthermore, GSH synthase inhibitor specifically enhanced the cytotoxicity of arctigenin, suggesting that intracellular GSH content appeared to be another factor to influence the susceptibility of cancer cells to arctigenin.

Finally, we evaluated the selective cytotoxicity of 9 herbal plant extracts in single or mixed doses. A combination of lower doses of these extracts significantly increased the cytotoxicity to cancer cells with no adverse effect on normal cells suggesting that selectivity against cancer cells was enhanced and toxicity to normal cells was reduced by interactions between the wide arrays of compounds in the mixed formulations. We further tested for the positive or negative interactions between these crude extracts and arctigenin. These observations indicated that combination of Kampo medicine can optimize specifically cytotoxicity to cancer cells while normal cells experience minimal toxicity.

In conclusion, this study clearly demonstrated that optimization of Kampo medicine can be achieved by appropriate combinations. Approaches based on interactions between Kampo-derived individual compounds or herbal plant extracts are necessary to find the optimum Kampo formulation for cancer treatment, and may open a new avenue for cancer chemotherapy.

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APPENDIX

Appendix 1

A. Preparation of MTS Solution from CellTiter 96® A_{Queous} MTS Reagent Powder

The following protocol is recommended for the preparation of 21 ml of MTS Solution (sufficient for ten 96 well plates).

1. A light-protected container was selected or a container was wrapped with foil for MTS Solution preservation at -20°C.
2. 21ml of DPBS was added to the container
3. 42mg of MTS Reagent Powder was weighted out and then was added to DPBS
4. MTS Solution was mixed at moderate speed on a magnetic stir plate for 15 minutes or until the MTS is completely dissolved.
5. The pH of the MTS Solution was measured. The optimum pH is between pH 6.0-6.5. pH was adjusted to pH 6.5 with 1N HCl if pH of the solution is above pH 6.5.
6. The MTS Solution was filter-sterilized through a 0.2µm filter into a sterile, light-protected container.
7. The MTS Solution was stored at-20°C protected from light.

B. General Protocol for Use of CellTiter 96® A_{Queous} Assay Reagents

The following recommendations are for the preparation of reagents sufficient for one 96 well plate containing cells cultured in a 100µl volume.

1. The MTS Solution and the PMS Solution were thawed. It should take approximately 90 minutes at room temperature or 10 minutes in a 37°C waterbath to completely thaw the 20ml size of MTS Solution.

2. 2.0ml of MTS Solution from the amber reagent bottle was removed using aseptic technique and transferred to a test tube.
3. 100µl of PMS Solution was added to the 2.0ml of MTS Solution immediately before addition to the culture plate containing cells.
4. The tube was swirled gently to ensure complete mixing of the combined MTS/PMS solution
5. 20µl of the combined MTS/PMS solution was pipetted into each well of the 96 well assay plate containing 100µl of cells in culture medium.
6. The plate was incubated for 1-4 hours at 37^{o}C in a humidified, 5% CO_2 atmosphere.
7. The absorbance at 490nm was recorded using an ELISA plate reader.

Appendix 2

Experimental protocol of DNA concentration measurement by using Quant-iT^{TM} dsDNA HS Assay Kits (for use with the Qubit^{TM} fluorometer)

1. The number of 0.5 mL tubes will be needed for standards and samples were set up. In this study we only used thin-wall, clear 0.5 mL PCR tubes. Acceptable tubes include Qubit^{TM} assay tubes (500 tubes, Invitrogen Cat. no. Q32856) or Axygen PCR-05-C tubes (VWR, part number 10011-830)
2. The tube lids were labeled
3. The Quant-iT^{TM} working solution was made by diluting the Quant-iT^{TM} dsDNA HS reagent 1:200 in Quant-iT^{TM} dsDNA HS buffer. A clean plastic tube was used each time Quant-iT^{TM} working solution preparation. .
4. 190 µL of Quant-iT^{TM} working solution was loaded into each of the tubes used for standards.

5. 10 µL of each Quant-iT™ standard was added to the appropriate tube and mix by vortexing 2-3 seconds carefully for avoiding the bubbles.
6. Quant-iT™ working solution was loaded into individual assay tubes so that final volume in each tube after adding sample is 200 µL. Sample can be anywhere between 1 µL and 20 µL, therefore, each assay tube was loaded with a volume of Quant-iT™ working solution anywhere between 180 µL and 199 µL.
7. Each of samples was added to assay tubes containing the correct volume of Quant-iT™ working solution (prepared in step 6) and mixed by vortexing 2-3 seconds. The final volume in each tube should be 200 µL
8. All tubes were allowed to incubate at room temperature for 2 minutes.
9. Any key was pressed to turn on the Qubit™ fluorometer.
10. **HOME** was pressed, the ▲and ▼keys were used to highlight **Quant-iT™ DNA, HS**, and **GO** was pressed to initiate the assay.
11. On the calibration screen, **Run new calibration** was highlighted and **GO** was pressed.

    Running a New Calibration:

    - The tube containing Standard #1 was inserted in the Qubit™ fluorometer, the lid was closed, and GO was pressed
    - Standard #1 was removed
    - The tube containing Standard #2 was inserted in the Qubit™ fluorometer, the lid was closed, and GO was pressed
    - Standard #2 was removed
12. A sample tube was inserted into the Qubit™ fluorometer, the lid was closed, and GO was pressed.
13. The reading given by the Qubit™ fluorometer was recorded

14. Reading samples and recording values were continued until all samples were read.
15. The concentration of the original sample was calculated by using the equation supplied below.

    Concentration of sample = QF value x$\left(\frac{200}{x}\right)$

    Where:

    QF value = the value given by the Qubit^{TM} fluorometer

    x = the number of microliters of sample added to the assay tube

Appendix 3

Protocol of 96-well fluorescence plate reader staining (detection of caspase 3 activity) by using Magic Red^{TM} Caspase Detection Kit

1. Cells were cultured to a density optimal for apoptosis induction. Cell density in the cell culture flasks should not exceed 10^6 cells/mL. Cell cultivated in excess of this concentration may begin to naturally enter apoptosis due to nutrient deprivation or the accumulation of cell degradation products in the media. Optimal cell concentration will vary depending on the cell line used.
2. Cells were seeded at 2 X 10^5 cells/well and pre-incubated for 12 hours
3. Cells were induced for apoptosis and the same time, an equal volume of non-induced cells were culture for use as a negative control cell population.
4. Once induction is complete, 150 µL of each cell suspension was transferred to a black microtiter plate.
5. 5µL 31X MR-$(DEVD)_2$ solution was added directly to the 150 µL cell suspension.

   Preparation of 31X MR-$(DEVD)_2$ solution:

   - The lyophilized MR-$(DEVD)_2$ was reconstituted with DMSO to yield a 155X concentrate (200 µL DMSO was added to the 100-test vial).

- The vial was mixed by swirling or tilting, allowing the DMSO to travel around the base of the vial until completely dissolved. At RT the reagent should be dissolved within a few minutes.
- 155X MR-$(DEVD)_2$ was stored in aliquot at or below -20^{o}C.
- 155X MR-(DEVD)2 stock was diluted 1:5 in H_2O. The unused 155X stock was stored at or below -20^{o}C
- The vial was mixed by inverting or vortexing at RT.

6. The cells were mixed gently by aspirating and expelling the cells with a pipette (to minimize cell shearing, the tip of the pipette was cut back to enlarge the hole of the tip)
7. The cells were incubated for at least 60 minutes at 37^{o} C under 5% CO_2 and protected from light. As cells settle to the bottom, these were re-suspended gently approximately every 20 minutes to ensure the MR-$(DEVD)_2$ is evenly dispersed among all cells.
8. Fluorescence intensity of the red fluorescence Magic Red^{TM} fluorophore was measured by setting the microplate reader in optimal excitation and emission wavelength tandem of 592 nm and 628 nm respectively.
9. The samples were read.

Appendix 4

Protocol for extraction of RNA from cultured cells by using AquaPure RNA Isolation Kit

Sample Collection

Cells or other cells were collected in suspension and placed on ice. The number of cells was determined using a hemacytometer or other cell counter. RNA was isolated as quickly as possible.

RNA Isolation from 1-2 million cultured cells, expected yield: 5-10 µg RNA

1. 1-2 million cells suspended in balanced salt solution or culture medium were added to a 1.5 ml microfuge tube on ice.
2. Cells suspension was centrifuged at 13,000-16,000 x g for 5 seconds to pellet cells. Supernatant was removed with a pipet leaving behind visible cell pellet and approximately 10-20 µl of residual liquid.
3. The tube was vortexed to resuspend the cells in the residual supernatant (tube was inverted to check that the cell pellet has disappeared completely). This greatly facilitates cell lysis in Step 4.
4. 300 µl RNA Lysis Solution was added to the resuspended cells and pipetted up and down no more than three times to lyse the cells.
5. 100 µl Protein-DNA Precipitation Solution was added to the cell lysate.
6. Tube was inverted gently 10 times and tube was placed into an ice bath for 5 minutes.
7. Tube was centrifuged at 13,000-16,000 x g in a microcentrifuge for 3 minutes. The precipitated proteins and DNA will form a tight white pellet.

8. The supernatant containing the RNA (leaving behind the precipitated protein-DNA pellet) was poured or pipeted into a clean 1.5 ml microfuge tube containing 300 µl 100% isopropanol.
9. The sample was mixed by inverting gently 25-30 times.
10. The sample was centrifuged at 13,000-16,000 x g in a microcentrifuge for 3 minutes; the RNA will be visible as a small, translucent pellet.
11. The supernatant was poured off and tube was drained briefly on clean absorbent paper. 300 µl 70% ethanol was added and the tube was inverted several times to wash the RNA pellet.
12. Tube was centrifuged at 13,000-16,000 x g in a microcentrifuge for 1 minute. The ethanol was poured off carefully.
13. The tube was inverted and drained on clean absorbent paper and sample was allowed to air dry 10-15 minutes.
14. 50 µl RNA hydration solution were added into the tube
15. RNA was allowed to rehydrate for at least 30 minutes on ice. Alternatively, RNA sample was stored at -70^{o}C to -80^{o}C until use.
16. Before use, sample was vortexed vigorously for 5 seconds and pulse spin. Sample was pipetted up and down several times to insure adequate mixing.
17. Purified RNA sample was stored at -70^{o}C to -80^{o}C.

Appendix 5

Protocol for total RNA quality check by using MultiNA machine

Preparation of samples and kit solutions for RNA analysis using MultiNA machine

1. 99 µl of TE buffer was added into 0.2 ml tube containing 1 µL of SYBR green II
2. The tube was vortexed for 15 seconds.
3. 5 µl of DEPC water was applied into 0.2 ml tube containing 1 µL of RNA 6000 Ladder and the mixture was mixed by pipetting.
4. The solutions as described in table below were mixed in 5 ml buffer tube for MultiNA machine.

Number of samples (including Blank and Ladder)	Separation Buffer (µL)	Diluted SYBER Green II Solution (µL)	Formamide (µL)
6	395	5	100
7~15	632	8	160
16~22	790	10	200

5. After closing the tube cap, the mixture solution was vortexed for 15 seconds and then set on the pink color holder of MultiNA main machine after opening the tube cap.
6. Total RNA solution was diluted to reach the concentration range 25-500 ng/µL

7. Blank, ladder and total RNA sample were prepared together according the certain volume as described in the table below

Type of reagent	Blank (µL)	Ladder (µL)	Total RNA sample (µL)
DEPC water (RNase-free water)	4.5		
Diluted ladder solution		4.5	
Total RNA solution (25-500 ng/µL)			4.5
RNA marker solution	4.5	4.5	4.5

8. Ladder and samples were boiled at 65^{o}C for 5 minutes, continued at 4^{o}C for 5 minutes on the thermal cycler.
9. After opening the caps, 8 well-tubes were set on the sample stand of MultiNA main machine.
10. Sample stand was covered using sample folder.

Steps for setting up of MultiNA machine

1. Main machine was switched on
2. The green light blinks on and off of MultiNA machine was waited before switching on the PC.
3. The software of MultiNA was opened
4. Milli-Q water was filled into glass bin for washing step (1 L/120 analyses) and prewashing
5. Glass bin was set for waste liquid with waste liquid tube.
6. Step of probe washing was done for 2 minutes
7. Step of chip washing was done for 20 minutes
8. MultiNA software was set up only for chip 4 using for RNA analysis

9. The sample sheets was managed on the MultiNA software
10. All tubes consisted of RNA Separation Buffer, Blanks, Ladder and total RNA samples were organized on the machine
11. The top cover of MultiNA main machine was closed and analysis process was started

Appendix 6

Procedure to synthesize single-stranded cDNA from total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems)

1. Each sample was prepared up to 2 µg of total RNA per 20 µL reaction.
2. The RT reaction mix was prepared using the kit components before preparing the reaction plate. To prepare the RT reaction mix (per 20 µL reaction):
    a. The kit components were allowed to thaw on ice
    b. The volume of components needed to prepare the required number of reactions were calculated referring to the following table:

Component	Volume/Reaction (µL)	
	+RT	-RT
2x RT Buffer	10.0	10.0
20x RT Enzyme Mix	1.0	-
Nuclease-free H2O	Q.S.† to 20 µL	Q.S.† to 20 µL
Sample	Up to 9 µL	Up to 9 µL
Total per Reaction	20.0	20.0

3. cDNA RT reactions were prepared to reverse transcription reactions:
    a. Aliquot 20 µL of RT reaction mix into tube
    b. The tubes were sealed
    c. The tubes were briefly centrifuged to spin down the contents and to eliminate any air bubbles
4. The tubes are placed on ice until these are ready to load the thermal cycler

5. Reverse transcription was performed by using the thermal cyclers with program setting according to the following table

	Step1	**Step2**	**Step3**
Temperature (^{o}C)	37	95	4
Time (min)	60	5	∞

6. Reaction volume was set to 20 µL
7. The reactions were loaded into the thermal cycler
8. The reverse transcription was started to run
9. cDNA RT tubes can be stored at 2^{o} to 8^{o}C for short-term or -15^{o}to -25^{o}C for long-term of storage duration.

Appendix 7

Procedures for real time – PCR analysis by using the Fast SYBR®Green Master Mix.

1. Prior to use, the Fast SYBR®Green Master Mix was mixed thoroughly by swirling the bottle
2. The volume of each component needed for all the wells in each assay was calculated based on the three replicates of each reaction.
3. The tubes were capped
4. The tubes were vortexed briefly to mix the solutions
5. The tubes were centrifuged briefly to spin down the contents and eliminate any air bubbles from the solutions
6. The appropriate volume of reaction mixture was transferred to each well of a plate (10-µL)
7. The plate was centrifuged briefly
8. Fast mode was selected to run the PCR reaction plate
9. The thermal cycling conditions was confirmed specified in the following table

Instrument	Step	Temp (^oC)	Duration (sec)	Cycles
StepOneTM	Enzyme activation	95	20	Hold
StepOnePlusTM	Denature	95	3	40
7500 Fast	Anneal/Extend	60	30	

10. In the plate document, the mode and enter the correct sample volume (10 µL)
11. The results were analyzed

Appendix 8

Protocol for protein extraction by using PRO-PREP^{TM} Protein Extraction Solution (iNtRON Biotechnology, Korea)

1. Suspension cell in 50 mL tube was centrifuged at 2,000-3,000 rpm for 5 min. Then cells were washed with PBS/DPBS. After washing, cells were counted and used approximately 5 x 10^6cells and then were transferred to the new 1.5 mL tube.
2. The cell pellet was harvested by centrifuged at 13,000 rpm for 10-20 seconds. After centrifugation, the remnant was removed using a pipette.
3. Cells were resuspended in 400 µL PRO-PREP^{TM} solution, and mixed well
4. Cell lysis was induced by incubation for 10-20 min on ice or freezer at -20^{o}C.
5. Suspension cell was centrifuged at 13,000 rpm (4^{o}C) for 5 minutes, and supernatant was transferred to a fresh 1.5 mL tube.
6. Protein concentration was measured.

Appendix 9

Protocol for analysis of protein quantitation by using Quant-iT^{TM} Protein Assay Kit (Invitrogen, USA):

1. The lids of the assay tubes were labeled. It will be needed for the standards and user samples.
2. The Quant-iT^{TM} protein working solution was made by diluting the Quant-iT^{TM} protein reagent 1:200 in Quant-iT^{TM} buffer.
3. Assay tubes were prepared according to the following table.

	Standard assay tubes (µL)	**User sample assay tubes (µL)**
Volume of working solution (from step. 2) to add	190	180-199
Volume of standard (from kit) to add; [prepare standard #1 by diluting 10 µL of the 0 ng/ µL standard, and standard #3 by diluting 10 µL of the 400 ng/ µL standard]	10	-
Volume of User Sample to add	-	1-20
Total volume in each assay tube	200	200

4. All tubes were vortexed for 2-3 seconds.
5. The tubes were incubated for 15 minutes at room temperature
6. The Qubit^{TM} fluorometer was calibrated using standard #1, standard#2, and standard #3
7. The user samples were read in the Qubit^{TM} fluorometer.
8. To determine concentration of the original sample, the value from the Qubit^{TM} fluorometer was multiplied by the dilution factor.

Appendix 10

Protocol for transferring protein into PVDF membranes by using the iBlot^{TM} Dry Blotting System (Invitrogen, Tokyo, Japan)

1. The lid of the device was opened
2. Sealing was removed and placed the anode stack, bottom with the tray directly on the blotting surface, aligned to Gel Barriers on the right.
3. Pre-run gel was placed on the transfer membrane of the anode stack
4. The pre-soaked iBlot^{TM} Filter Paper (soaked in deionized water) was placed on the pre-run gel and remove air bubbles using the Blotting Roller.
5. The sealing of the Cathode Stack was removed, the red plastic was discarded
6. The Cathode Stack was placed, Top over the pre-soaked Filter paper with the electrode side facing up and aligned to the right edge. Air bubbles were removed using the Blotting Roller.
7. The Disposable Sponge was placed with the metal contact on the upper right corner of the lid.
8. Blotting was performed within 15 minutes of assembling the stacks with the gel through some following steps:
    a. The lid was closed and secures the latch. The red light is on indicating a closed circuit.
    b. The correct program was ensured and time is selected.
    c. The start/stop button was pressed. The red light changes to green.
    d. Current automatically shuts off at the end of each run. The end of transfer is indicated by beeping sounds, and flashing red light and digital display. The start/stop button was pressed. The light turns to a steady red.