Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2016; 8(5); 841-847

ISSN: 0975-4873

Research Article

Studies on the Enhancement of Cancer-Selective Cytotoxicity of Herbal Medicine by Combination

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Available Online: 1st May, 2016

ABSTRACT

In this study, we evaluated the selective cytotoxicity of 9 herbal plant extracts in single or mixed doses by using a panel of normal and cancer cell lines. Single and higher doses of herbal extracts selectively suppressed 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, the compound responsible for cancer-selective cytotoxicity in extracts of the herb *Arctium lappa* L. 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 suggest that combination of herbal medicine can optimize cytotoxicity specifically to cancer cells while normal cells experience minimal toxicity. This study thus describes the optimization of herbal formulations through interactions between herbal plant extracts, and discusses the importance of this approach for enhancing cancer-specific cytotoxicity and opening a new avenue for cancer chemotherapy.

Keywords: Cancer**,** Herbal, Cytotoxicity, Combination.

INTRODUCTION

Kampo medicine (KM) has its origin in traditional medicine in China from where it, spread to Japan in the fifth and sixth centuries[1]. 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[2]. 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[3-9]. Cancer has been the world's leading killer disease[10,11]. Various treatment strategies have been employed, such as chemotherapy, surgery, radiation, and immunotherapy using monoclonal antibodies[12]. Although chemotherapy is the most common choice for cancer treatment, its drawbacks are the severe side effects[13]. These reduce the quality of life of cancer patients, and they have been a major issue in cancer treatment[14,15]. We have been working on the isolation of anti-cancer agents with low side effects from medicinal or herbal plants[16-19]. 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[20]. 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. 1) as an active component of cancer- selective cytotoxicity from the medicinal herb *Arctium lappa*. L.[19]. Arctigenin specifically inhibited the growth of lung cancer cells A549, and induced apoptosis with no adverse effect on normal cells[19]. 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[21]. Empirical observations of traditional practices prove that some herbal plant extracts applied as a composite formula provided an effective cure[22]. 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 in vitro as single dose or as composite formulation prior to clinical trials[23]. The aim of this study was therefore to test the efficacy of the combinations of herbal extracts for their

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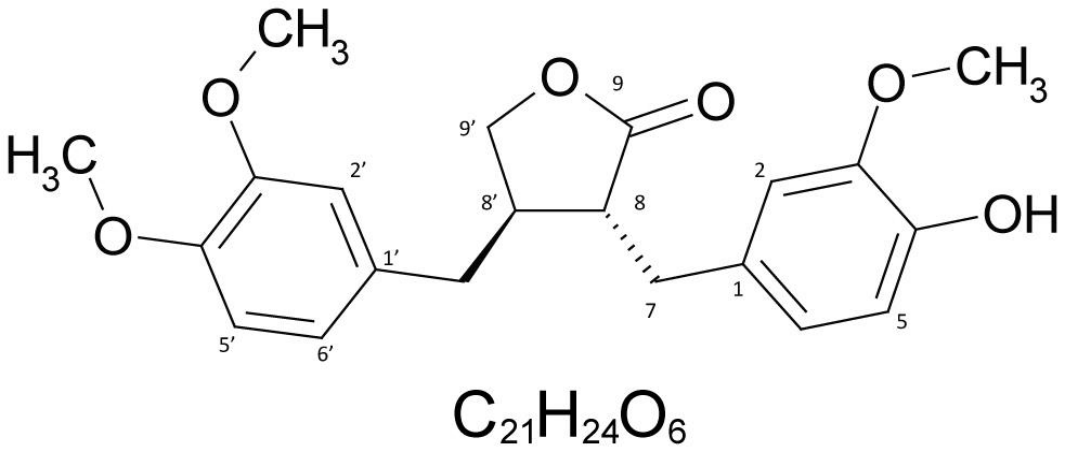

Figure 1: The chemical structure of arctigenin identified as the cancer-selective cytotoxic component of the extract from *Arctium lappa* L.

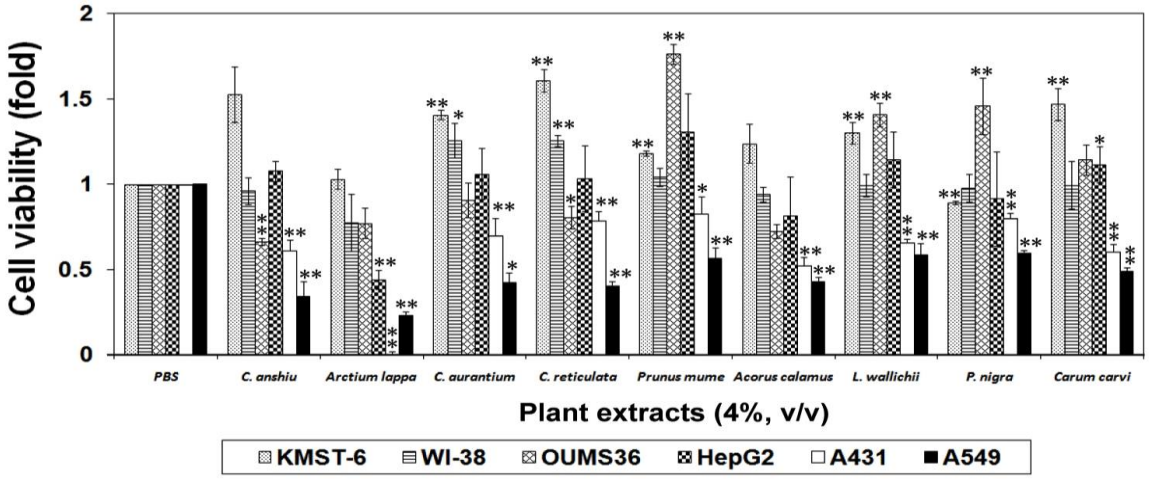

Figure 2: Viabilities of various cancer cells after exposure to 4% dose of 9 herbal extracts. The normal cells used were KMST-6, WI-38, and OUMS-36T-5F. The cancer cells used were HepG2, A431, and A549. For the descriptions of these cells, refer to the Materials and Methods section. Cells seeded at a 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).

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.

MATERIALS AND METHODS

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□m) and stored at 6°C in a refrigerator. They represents 100% extract concentration.

Cell Culture

The various normal or cancerous human cell lines used for the antitumor assay listed 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 37^0C.

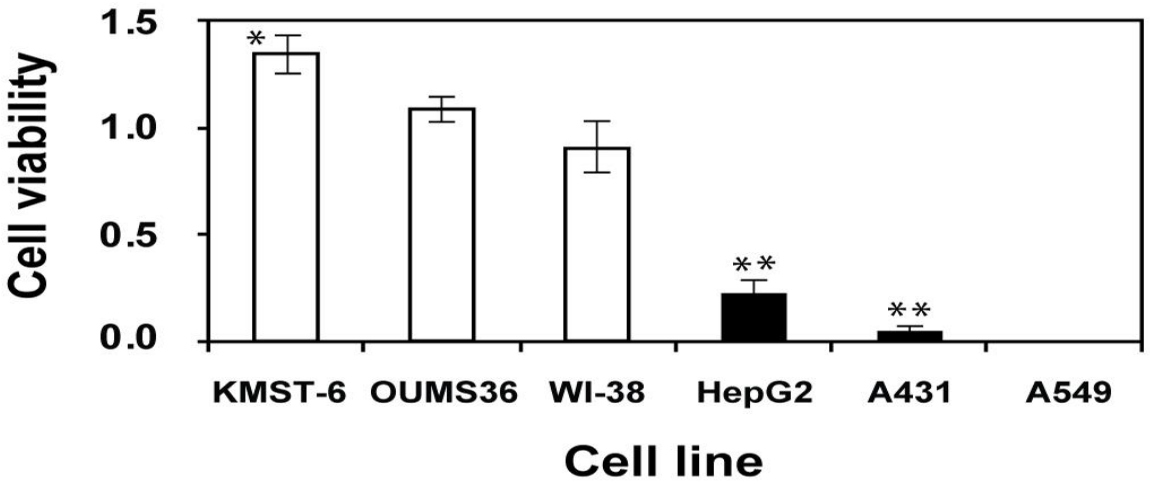

Figure 3: Viabilities of cancer and normal cells after exposure to the composite formula of 9 herbal plant extracts. The normal cells used were WI-38, OUMS-36T-5F, and KMST-6. The cancer cells used were A549, HepG2, and A431. For a more detailed description of these cells, refer to the Materials and 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 of triplicate analyses. *p < 0.05; **p < 0.01 versus control (PBS).

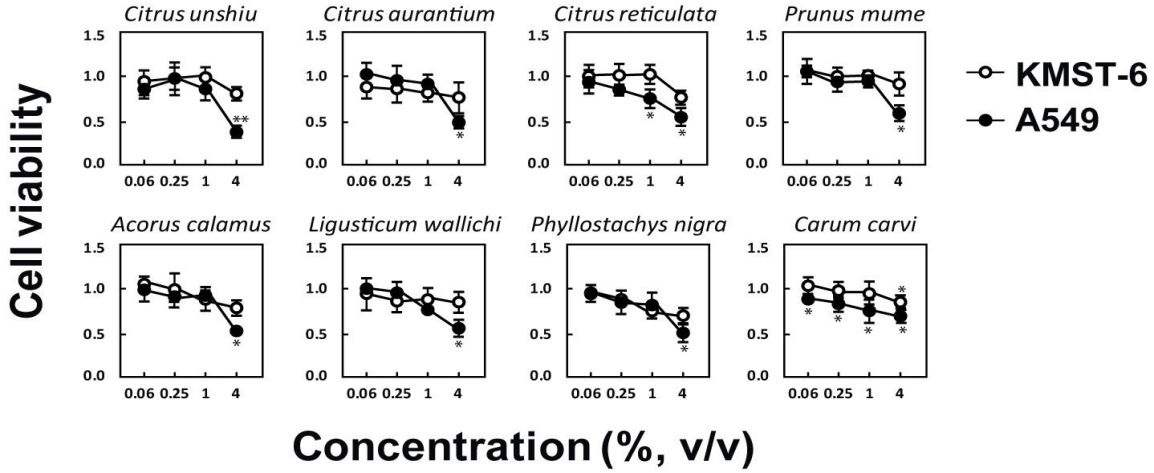

Figure 4: Viabilities of A549 (cancer) and KMTS-6 (normal) cells after exposure to the various concentrations of herbal plant extracts. Open and closed circles show viabilities for normal and cancer cells, respectively. Cells seeded at a density of 1,000/well were pre-cultured for 24 h, and treated with 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.

Cytotoxicity Test

Cell viability was measured using the MTS assay kit according to the manufacturer's instructions (Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega Co, Madison, USA). 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.

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 were used: 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).

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.

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.

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.

RESULTS

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. 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 to that from *A. lappa. L.*

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 that were, 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. 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.

Combination of arctigenin with crude extract

Fig. 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[19], 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. 1). Arctigenin specifically lowered the viability of cancer cells with no adverse effect on the normal cells[19]. 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 in vitro. 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 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*. 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.*

DISCUSSION

Table 1: ED_{50} (%) of extracts against A549 and KMST-6 cells in the presence (+) or absence (-) of arctigenin

Plant Extracts	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 fibroblastcell

The present study confirmed the cancer-selective cytotoxicity of 9 herbal plant extracts chosen from 364 specimens screened in our previous study[19], 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[22,24]. 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.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. 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[25-27]. 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[22]. Purification of the selective cytotoxic activity in the extract of *A. lappa.* L. resulted in the identification of arctigenin as the active component (Fig.1). Arctigenin is a phenylpropanoid dibenzylbutyrolactone lignan with antioxidant, anti-inflammatory and antitumor activities[28-30]. 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 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 in the combination with arctigenin, and further optimization may be necessary to minimize the cytotoxicity to normal cells. As shown in Fig. 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 of 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.

ACKNOWLEDGEMENT

We would like to thank Dr. Seikoh Saitoh for his technical assistance in the estimation of the ED_{50} value with the R-driven program as described in the Materials and Methods section.

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