Combined Treatment of Herbal Mixture Extract H9 with Trastuzumab Enhances Anti-tumor Growth Effect

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Introduction

Breast cancer, which is the most frequent type of cancer affecting women in the world, is characterized by multiple molecular phenotypes [6, 7, 10, 14, 16]. Because breast cancer patients within clinically and morphologically similar classes show varied response to therapy, the traditional prognostic factors currently available are insufficient for patient classification. Thus, the detailed molecular classification of breast tumors is being elucidated to explain the genetic heterogeneity of breast tumors, and to develop appropriate therapeutic strategies. In general, the following four distinct molecular breast cancer groups were identified based on gene expression profiles [28]: luminal epithelial/estrogen receptor (ER) positive, c-erb-B2 (HER2) positive, basal-like, and normal breast-like. A subsequent study divided the luminal/ER-positive group into three subtypes: luminal A, B, and C [33], but existence of the luminal C group remains uncertain [34].

The combination adjuvant chemotherapy of doxorubicin and cyclophosphamide (AC) is considered to be the most effective combination chemotherapy for breast cancer treatment [22]. These conventional chemotherapeutic drugs are used without molecular classification of the patients and often lead to adverse side effects by causing unnecessary damage to normal and healthy cells. Thus, more selective therapeutic strategies are under development. Selective estrogen receptor modulators, including tamoxifen and raloxifen, are used to treat ER-positive patients, whereas the HER2-targeted monoclonal antibody drug, trastuzumab,
is used on HER2-positive patients [27]. On the other hand, to overcome the adverse effects of chemotherapy, extracts from medicinal herbs may also be used to give additive or synergistic preventive effects for the inhibition of tumor growth [3]. The combined treatment of chemotherapeutic drugs with herbal extracts could lower the concentration of chemotherapeutic drug required to show the same anticancer effects [26].

Herbal extracts exhibit a variety of activities, including anti-allergic, antimicrobial, anti-oxidant, and anticancer effects, due to the presence of various components [2], and usually exert cytotoxic effects on tumor cells directly, or through enhancement of the immune response. For this reason, the anti-tumor activity of various herbal extracts is currently being studied [5, 32]. In the present study, the most effective herbal extracts were screened, and their combined effects with chemotherapeutic drugs and a targeted drug were examined. To determine the molecular mechanism underlying the anti-tumor growth effects, the immune cell population was also analyzed.

Materials and Methods

Cell Cultures
The human breast cancer cell lines MDA-MB-231 and MCF-7, as well as the murine breast cancer cell line 4T1, were obtained from the American Type Culture Collection (USA). The human fibroblast cell line HFCH8 was a kind gift from Dr. Myeong-Sok Lee (Sookmyung Women’s University, Korea). MDA-MB-231, MCF-7, and HFCH8 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, USA) at 37°C in a humidified 5% CO2 incubator as described previously [19]. The 4T1 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS.

Oriental Medicinal Herbs and Formulation of H5, H9, and H11
The medicinal herb ingredients included in H5, H9, and H11 are listed in Table 1. The herbal ingredients were obtained from the Oriental Medical Hospital, Dongguk University (Korea), and were kindly authenticated by Dr. Seong Hyun Jung (College of Oriental Medicine, Dongguk University). Ethanol extracts from the listed plants were prepared as follows. The dried and pulverized medicinal herbs were mixed together, and the indicated amounts of each herb were mixed, soaked in 10× (w/v) of 40% ethanol, and then extracted for 3 h at 95°C. The ethanol extracts were concentrated using a rotary evaporator, lyophilized, and then reconstituted in distilled water for the in vitro studies.

Cell Viability Assay
Cell proliferation was measured by direct cell counting. Briefly, MDA-MB-231, MCF7, HFCH8, and 4T1 cells were collected and seeded at a density of 5 × 104 cells/well in 24-well plates, and then treated with various concentrations of H5, H9, and H11. After incubation for 48 h, cells were trypsinized, and the viable cells were counted using a hemacytometer after trypan blue staining to exclude dead cells, as described previously [20].

Animals
All animals were obtained from Harlan Laboratories (USA). Six-week-old Balb/c female mice were subdivided, acclimatized for one week, and divided randomly into experimental groups. Bedding was changed once a week, and the temperature and humidity were controlled. Mice were housed under 12 h light/12 h dark conditions and allowed free access to food and water. The plans and protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of Sookmyung Women’s University, Seoul, Korea.

Syngeneic 4T1 Tumor-Bearing Mice Model
4T1 cells at a concentration of 2 × 105 per 0.1 ml of PBS were injected subcutaneously into mice, and tumor growth was monitored by measuring the tumor volumes with calipers [Volume = (length

<table>
<thead>
<tr>
<th>Table 1. The compositions of H5, H9, and H11.</th>
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<tr>
<td>H5 (%)</td>
</tr>
<tr>
<td>Cyperus rotundus L. (22.2)</td>
</tr>
<tr>
<td>Evodiae Fructus (22.2)</td>
</tr>
<tr>
<td>Psoraleae Semen (22.2)</td>
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<tr>
<td>Sparganii Rhizoma (22.2)</td>
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<tr>
<td>Curcumae Radix (11.1)</td>
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<tr>
<td>Myristicae Semen (12)</td>
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<tr>
<td>Alpiniae Officinar Rhizoma (8)</td>
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<tr>
<td>Sparganii Rhizoma (4)</td>
</tr>
<tr>
<td>Curcumae Radix (12)</td>
</tr>
<tr>
<td>Glycyrrhize Radix et Rhizoma (9)</td>
</tr>
<tr>
<td>Aconiti Lateralis Radix Preparata (9-18)</td>
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H9 and H11 were administered daily at a concentration of 400 mg/kg by oral injection. For the vehicle treatment group, the same volume of distilled water was administered. Trastuzumab was administered every third day at a concentration of 1 mg/kg by intraperitoneal injection, and IgG (Sigma, USA) was given as an experimental control. For AC treatment, adriamycin (Sigma, USA) and cyclophosphamide (Sigma, USA) were mixed and administered intraperitoneally once, 7 days after tumor injection. PBS was given as a vehicle treatment.

**Flow Cytometry Analysis**

Splenocytes were separated from cell debris using a 70 µm nylon cell strainer (BD Bioscience, USA) after removal of red blood cells (RBC) using RBC lysis buffer (Sigma, USA). The prepared splenocytes were stained in phosphate-buffered saline with 1% FBS for 30 min at 4°C with the following fluorescein isothiocyanate-, phycoerythrin-, PECy7-, or allophycocyanin-conjugated anti-mouse antibodies: anti-CD3ε, -DX5, -CD8, -CD4, -CD11b, and -Gr1. All antibodies were purchased from eBioscience (USA). The labeled cells were analyzed on a FACS Canto II cytometer equipped with FACS Diva software (BD Bioscience, USA), and data were analyzed using FlowJo software (USA).

**NK Cell Purification and Cytotoxicity Assay**

NK cells were isolated from freshly obtained mouse splenocytes using an NK cell isolation kit (Miltenyi Biotec, Germany), according to the manufacturer’s protocol. For NK cell cytotoxicity assay, viable NK cells were counted with the trypan blue dye exclusion method and used as effectors in the cell-mediated cytotoxicity assay. YAC-1 target cells were used as target cells, and the NK cells were distributed in triplicate at the effector-target cell ratios from 5:1 to 1:1. The cells were incubated for 3 h at 37°C in 5% CO2. At the end of the incubation, dead target cells were labeled with fluorescence labeled caspase-3 (BD Bioscience, USA), and the percentage of YAC-1 cell death was analyzed on a FACS Canto II cytometer equipped with FACS Diva software. Data were analyzed with FlowJo software, as described previously [9].

**Statistical analysis**

The paired Student’s t-test and one-way factorial ANOVA were employed for statistical analysis, along with the Tukey and Scheffe tests. Values of $p < 0.05$ were considered as significant differences. Data were presented as the mean ± standard deviation (SD).

**Results**

**Selection of Effective Herbal Extracts with Anti-Breast Cancer Activity**

*Osyubujaijingy-tang* described in *Donguisusebowon* was the typical prescription used to cure *Shao Yin* (Lesser Yin) disease. The hepatoprotective effects of *Osyubujaijing-tang* were demonstrated in a chronic liver cirrhosis model [36], and it was also shown to improve the symptoms of peripheral T-cell lymphoma patients, including fever, myalgia, poor performance status, neck pain, and headache [13]. The extracts of each herb in *Osyubujaijing-tang* also have anti-tumor effects. Crude extract of Cinnamomi cortex showed anti-tumor activity against Sarcoma 180 in mice, and suppressed adjuvant-induced arthritis in rats [30]. Crude extract of Evodiae Fructus is known to inhibit the proliferation of several tumor cell lines and to induce tumor cell death through the caspase pathway [8]. Crude extract of *Alpinia officinarum* rhizomes is known to be a viable therapeutic or preventive candidate for the treatment of acute and chronic arthritis patients [18]. In addition, crude extract of Curcumae Radix is a potential chemopreventive agent for gastric cancer, because it suppresses the expression of VEGF, COX-2, and PCNA in the gastric mucosa of rats [21]. These facts stimulated us to examine whether *Osyubujaijing-tang* has anti-tumor growth effects.

Ethanol extraction was used instead of water to extract the many active ingredients from *Osyubujaijing-tang*. The ethanol extract of *Osyubujaijing-tang* was named H11. To make a new extract with the highest anticancer efficacy against breast cancer, the additional herbal extracts H5 and H9 were designed. Each component of H5 and H9 was selected based on its ability to induce apoptosis and immune activation from H11 (Table 1).

**H5, H9, and H11 Inhibit the Proliferation of Breast Cancer Cells**

To evaluate the effects of the herbal extracts on the proliferation of breast cancer cells, breast cancer cell lines, including human MDA-MB-231 and MCF-7 cells, as well as mouse 4T1 cells, were treated with H5, H9, and H11 at the indicated concentrations for 48 h. The viable cells were then counted using a hemocytometer after trypan blue
staining to exclude dead cells. All three extracts were observed to inhibit the proliferation of breast cancer cell lines. Interestingly, H5 demonstrated the strongest inhibition of proliferation of all three cell lines at the concentration of 0.5 mg/ml, and H9 also showed stronger inhibition than H11 (Figs. 1A–1C). It is well known that herbal extracts that demonstrate anti-proliferative ability in vitro sometimes fail to suppress tumor growth in vivo. Thus, the ability of the three extracts to suppress tumor growth was next examined in vivo. The mouse breast cancer cell line 4T1 was subcutaneously injected into the mammary pad of female Balb/c mice. Daily oral administration of the herbal extracts was then carried out from 3 days after 4T1 injection. Although H5 showed the highest effectiveness for inhibition in vitro, the anti-tumor growth effects of both H9 and H11 were superior to H5 in vivo (Fig. 1D). Thus, the remaining investigation focused on H9.

**H9 Treatment Induces Apoptosis in Breast Cancer Cells**

To determine the mechanism underlying the anti-tumor growth effect of H9, the morphological changes of 4T1 cells were photographed after the H9 treatment for 24 and 48 h (Fig. 2A). The untreated cells appeared to be intact, whereas cell shrinkage resembling an apoptotic phenotype was observed after H9 treatment. Thus, to determine whether the cell death was related to apoptosis, the levels of expression of apoptotic markers were examined. The apoptotic markers cleaved poly-ADP ribose polymerase
(PARP) and caspases, as well as the anti-apoptotic marker Bcl-2, were examined after treatment with H9 at the indicated concentration for 48 h. Cleavage of PARP, caspase 3, and caspase 9 was observed after 48 h of treatment with 150 µg/ml H9, whereas decrease of the anti-apoptotic protein Bcl-2 was observed (Fig. 2B). This finding implies that H9 directly induces apoptosis in 4T1 cells. To know whether the H9-induced apoptotic effect is specific to tumor cells, HFCH8 cells (a normal human fibroblast cell line) were treated with H9 for 48 h and no apoptotic cells were observed, unlike breast cancer cells. Moreover, apoptotic and anti-apoptotic proteins were not altered (Figs. 2C–2D). These results indicate that the apoptotic effect of H9 is more selective to breast cancer cells than normal fibroblast cells.

**H9 and H11 Do Not Show Combined Effects with Chemotherapeutic Drugs**

Herbal extracts are generally used as an adjuvant therapy with anticancer drugs to relieve the pain or to enhance the cytotoxicity of the anticancer drugs and fight tumor immunity. To examine the combined effects of H9 and H11 with chemotherapeutic drugs, adriamycin and cyclophosphamide (AC) were chosen for analysis, because these two drugs are commonly used as an anticancer drug cocktail for the treatment of breast cancer patients. The 4T1 cells were injected into the mammary pad of female Balb/c mice and AC was administered intraperitoneally once, 7 days after 4T1 injection. Oral administration of H9 and H11 was carried out daily after an additional 7 days. Tumor growth was measured every other day, and the data

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**Fig. 2.** H9-induced apoptosis of breast cancer cells. 
(A) 4T1 cells were treated with the indicated concentrations of H9 for 24 and 48 h. Representative photographic images are shown. (B) 4T1 cells were treated with the indicated concentrations of H9 for 48 h, after which cell lysates were prepared. The levels of PARP, caspase 3, caspase 9, and Bcl-2 were examined using immunoblot assay. (C) HFCH8 cells were treated with the indicated concentrations of H9 for 24 and 48 h. Representative photographic images are shown. (D) HFCH8 cells were treated with the indicated concentration of H9 for 48 h and then the levels of PARP and Bcl-2 were examined using immunoblot assay.
were used for generation of a graph (Fig. 3A). On the 18th day after treatment with herbal extracts, the mice were sacrificed and the tumor masses were weighed and photographed (Figs. 3B–3C). The combined treatment of AC with herbal extract did not show enhanced anti-tumor effects, although separate treatment with H9 and H11 also showed anti-tumor growth effects, respectively. However, it cannot be ruled out that the effect of AC was too strong to show the combined effect with H9 and H11.

**Combined Treatment of H9 and Trastuzumab Shows the Most Remarkable Anti-Tumor Growth Effect**

Because the combined treatment of AC with H9 and H11 did not show enhancement of the anti-tumor growth effects, examination was carried out to determine whether H9 and H11 could show additive anti-tumor growth effect with trastuzumab, a HER2-targeting monoclonal antibody. The 4T1 cells were injected into the mammary pad of female Balb/c mice and trastuzumab was administered intraperitoneally every third day from 7 days after 4T1 injection. After an additional 7 days, H9 and H11 were orally administered daily. Tumor growth was measured every other day and data were used for the generation of a graph. Trastuzumab treatment significantly suppressed tumor growth, while the combined treatment of trastuzumab and H9 showed the most effective suppression. The tumor weight was also greatly reduced in the trastuzumab and H9 combination treatment, without body weight change (Figs. 4A–4C). In general, tumor-bearing mice showed increased spleen weight because of immune activation. The H9 treatment reduced the spleen weight in 4T1 tumor-bearing mice (Fig. 4D), indicating the possibility that H9 could regulate immune cell function.

**Combined Treatment of H9 and Trastuzumab Shows Increased Natural Killer (NK) Cell Population**

To determine the mechanism underlying the anti-tumor growth effects of the combined therapy of H9 and
trastuzumab, the immune cells with anti-tumor function were analyzed. The 4T1 tumor-bearing mice were subjected to H9 and trastuzumab treatment, as in Fig. 4A, and then the immune cells were analyzed using harvested spleens after sacrifice of the mice. The population of NK cells was increased from 3% at basal level to about 6% by the H9 and trastuzumab combination treatment (Figs. 5A–5B). To determine whether the increased NK cells had enhanced NK cytotoxicity, the NK cytotoxicity was examined. The NK cells isolated from spleen and Yac-1 target cells were mixed together at the indicated ratio and incubated for 3 h to allow the NK cells to kill the Yac-1 target cells. The combined treatment of H9 and trastuzumab indeed increased the cytotoxicity of the NK cells (Fig. 5C).

In addition to NK cells, the CD8-positive T-cell population was also examined. This population was not affected by the combined treatment of H9 and trastuzumab (Figs. 5D–5E). However, analysis of the MDSC population demonstrated great reduction by the combined treatment of H9 and trastuzumab (Figs. 5F–5G). The results from the immune cell profile analysis indicate that the combined treatment of H9 and trastuzumab decreased the immune-suppressing MDSC while increasing tumor-killing NK cells.

Discussion

Traditional medicinal herb extracts usually exert their cytotoxic effects on tumor cells directly, or by enhancement of the immune response. Herbal extracts can also lower the cytotoxic effects of conventional chemotherapy drugs. In
Fig. 5. Combined effects of H9 and trastuzumab on anti-tumor immune cells, including NK, CTL, and MDSC cells.

(A, B) NK cell population (CD3-/DX5+) among splenocytes was analyzed by fluorescence-activated cell sorting analysis and shown as a dot blot (A) and bar graph (B). (C) Ex vivo NK cell cytotoxicity assay was performed. (D, E) The CD8+ T-cell population (CD3+/CD8+) among splenic lymphocytes was analyzed by fluorescence-activated cell sorting analysis and shown as a dot blot (D) and bar graph (E). (F, G) MDSC population (Gr-1+/CD11b+) among splenocytes was analyzed by fluorescence-activated cell sorting analysis and shown as a dot blot (F) and bar graph (G). Splenocytes from mice were stained with the indicated antibodies toward cell surface markers and analyzed by flow cytometry. *, p < 0.05. **, p < 0.01 by ANOVA.
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this study, H9 showed the most effective anti-tumor activity against breast cancer cells in vitro, although H5 was the most effective in vitro. Because several components of H9 have anticancer effects [12, 17, 35], it is conceivable that the total H9 extract would show anti-tumor activity. The marker compounds of H9 are evodiamine, ginsenosides Rg1 and Rb1, and cinnamic acid. Gas chromatography/mass spectrophotometry showed that the H9 extract contained coumarin, isoeugenol, isoeleminic, angelicin, and psolaren as the major compounds. Angelicin, which is structurally related to psoralens, has apoptosis-inducing effect and is a well-known chemical class of photosensitizers, used for its anti-proliferative activity in the treatment of different skin diseases. Angelicin was also reported to be an effective apoptosis-inducing natural compound of human neuroblastoma cancer, and its analogs have been shown to have potent anti-influenza and anticancer activities [29, 31]. Thus, angelicin would be one of the major compounds conferring the anti-tumor activity of H9. Coumarin and its derivatives have multiple biological activities, such as antifungal, antiviral, anticancer, anti-inflammatory, and antidiabetic activities. Coumarin treatment also enhances the macrophage migration activity in the presence and absence of LPS and increased nitric oxide release. This suggests that the immunomodulatory activity of coumarin, a component of H9, would contribute to the anti-tumor effect by enhancing the anti-tumor immune function. Although the combined treatment of coumarin with cimetidine was reported to increase the percentage of monocytes without NK cell population change [11, 23, 24], it is possible that coumarin, along with other compounds present in H9 or trastuzumab, could be involved in the population change of NK cells and MDSC. Taken together, angelicin and coumarin may be the biologically active compounds in H9.

Although the molecular mechanisms of herbal extracts have not been well-characterized, the safety and efficacy of many medicinal herb extracts have been validated [1, 37]. Medicinal herb extracts can be used as a strategy to reduce the side effects caused by chemotherapy or to enhance the effects of chemotherapeutic drugs. Therefore, the combined effect of H9 and AC was examined in vivo, in addition to the anti-proliferative effect of H9 observed in vitro. Co-treatment of H9 and AC failed to show additive or synergistic anti-tumor growth effects. However, the lack of a combined effect may be due to the strong anti-tumor effect of AC treatment as a primary chemotherapy. Thus, the combined effect of H9 and trastuzumab was also examined. Trastuzumab, which is a HER2-targeted monoclonal antibody, is also used as an adjuvant therapy for breast cancer. The combination of H9 and trastuzumab was found to demonstrate enhanced anti-tumor effects through increase in the NK cell population and decrease in the MDSC population. On the other hand, it is possible that the MDSC population was decreased in H9/trastuzumab-treated mice because of the loss of tumor weight. Therefore, it is valuable to find out whether H9/trastuzumab directly suppresses MDSC differentiation from the precursor cells or MDSC expansion.

There are many immune modulating compounds in the H9 extract. Ginsenoside Rg1, one of the compounds in H9, increases the number of T helper cells and the splenocyte NK cytotoxicity. CML-1 oriental herbal extract containing Cinnamoni Cortex inhibits TNF-α-induced inflammation [15, 25]. Curcumol, one of the major components of the essential oil of Rhizoma Curcumae, inhibits lipopolysaccharide-activated RAW264.7 cells through the suppression of TNF-α, IL-1, IL-6, and iNOS [4]. In addition to these known immune modulating compounds, further study is needed to identify the compounds related to the change of NK cell and MDSC number and activity.

In summary, H9 inhibited the proliferation of breast cancer cells, eventually leading to apoptotic death. Furthermore, H9 suppressed 4T1 tumor growth in a syngeneic tumor model, and the combined treatment of H9 and trastuzumab further enhanced the anti-tumor growth effect through increase of the NK cell number and activity and decrease in the MDSC number. Although some of the compounds from the H9 extract have anti-tumor activity and immune modulating activity, exactly which compound of H9 was responsible for the synergistic effect with trastuzumab for inhibition of tumor growth remains to be elucidated.

Acknowledgments

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References


