Differential control of growth, cell cycle progression, and gene expression in human estrogen receptor positive MCF-7 breast cancer cells by extracts derived from polysaccharopeptide I'm-Yunity and Danshen and their combination

TZE-CHEN HSIEH and JOSEPH M. WU

Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595, USA

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Abstract. The use of herbs has been the mainstay of treatment for a variety of human illnesses and is an essential part of culturally-based healing traditions in many societies and countries. Also, herbs, including Chinese herbs, are being incorporated as remedies for disease management and treatment in Western countries. In Traditional Chinese Medicine (TCM), herbal prescriptions are most frequently given to patients as complex formulations containing multiple herbs. Notably and unwittingly, this approach amounts to the administration of several chemical entities at once; the underlying theory is that interactions among the chemicals present in different herbs in a formula exert synergistic pharmacodynamic actions and neutralize the adverse effects and toxicities of specific individual chemicals. The effectiveness and mechanisms of this approach have not been well investigated or understood. A primary interest of this laboratory is to obtain experimental evidence that supports the fundamental mechanistic theme for the combinatorial herbal strategy described above and its potential application in preventing and treating breast cancer (BCa). In this study, we investigated the effects of 70% ethanolic extracts prepared from medicinal mushroom extract denoted I'm-Yunity and Danshen (*Salvia miltiorrhizae* Binge), alone and in combination, using MCF-7 cells as an *in vitro* model of estrogen receptor positive (ER^+), low invasive BCa. Combination of I'm-Yunity and Danshen (referred to as I'm-Yunity-Plus) suppressed clonogenicity to a comparable degree as Danshen alone; both being significantly more active than I'm-Yunity. However, extract of Danshen was more active in inhibiting MCF-7 cell growth than I'm-Yunity-Plus. In comparison, I'm-Yunity elicited less growth inhibition. Flow cytometric analysis showed that I'm-Yunity-Plus induced partial block of G_1/S transition in MCF-7 cells, whereas Danshen slowed down cell progression from G_1/S into G_2/M phases of the cell cycle. Treatment by I'm-Yunity did not affect cell cycle progression in MCF-7 cells; however, it promoted active induction of apoptosis. In addition, treatment with Danshen alone resulted in a pronounced reduction in the expression of Rb, cyclin D1, and p53, and also led to a diminution of p65 and p50 forms of NF-κB. The pronounced suppressive effects of Danshen on expression of the aforementioned genes were largely attenuated in cells treated with I'm-Yunity-Plus suggesting that ingredients in Danshen must have interacted with those in I'm-Yunity as to culminate in neutralization of the gene suppressive effects of Danshen. Additional support for such interactions was obtained by targeted cDNA array analysis using human tumor metastasis and BCa/ER signaling gene arrays. Taken together, our results are consistent with the interpretation that interaction exists between Danshen and I'm-Yunity and that I'm-Yunity-Plus may have efficacy in the treatment of BCa, particularly for patients with ER^+ status.

Correspondence to: Dr Joseph M. Wu, Room 147, Department of Biochemistry and Molecular Biology, Basic Sciences Building, New York Medical College, Valhalla, NY 10595, USA
E-mail: joseph_wu@nymc.edu

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Introduction

Breast cancer (BCa) is the most common cancer among women in the US and other developed and also developing countries (1,2). There is consensus among clinicians and researchers that BCa is a preventable disease. Combination chemotherapies produce more clinically relevant, longer duration, better survival responses than single agent therapies and hence, have become the standard of management for metastatic, hormone-refractory BCa, and increasingly, also primary BCa. Unfortunately, they also produce significant side effects and long-term treatment can further be complicated by development of chemoresist tumor clones (3). Consequently, individuals diagnosed with BCa seek alternative treatment modalities including complementary and alternative medicine (CAM), to bolster the primary means of medical intervention and to alleviate their adverse effects. It has been estimated that as many as 50% of breast cancer patients use CAM, particularly biopharmacologic and herbal approaches (4).

Herbal medicine is one of the most ancient forms of health care known to humankind and has been used in many cultures throughout history (5-8). Typically, herbal remedies emphasize the use of whole extracts from a single herb or combined extracts from multiple herbs in the form of formulations, as is the practice among Chinese herbalists and Traditional Chinese Medicine (TCM) specialists (9,10). This method of disease management and treatment inadvertently enforces the administration of several chemical entities at once; the underlying theory is that interactions among the chemicals present in different herbs in a formulation likely will exert synergistic pharmacodynamic actions while simultaneously neutralize the adverse effects and toxicities of specific individual chemicals (11-13). In certain respects, therefore, herbal remedies resemble some of the same fundamental themes of chemoprevention, a concept originally introduced by Sporn in the 1970s advocating the use of natural or synthetic compounds to inhibit or retard cancer development (14-17). A major deficiency of the polyherbal formulation approach is that the effectiveness and mechanisms have not been well investigated or understood.

We sought to obtain experimental evidence that supports the fundamental mechanistic theme for the combinatorial herbal strategy described above and its potential application in preventing and treating BCa. In the present study, we investigated the effects of 70% ethanolic extracts prepared from medicinal mushroom extract denoted I'm-Yunity and Danshen (*Salvia miltiorrhizae* Binge), alone and in combination (combination of I'm-Yunity and Danshen is referred to as I'm-Yunity-Plus), using MCF-7 cells as an *in vitro* model of estrogen receptor positive (ER^+), minimally invasive BCa. I'm-Yunity is a family of distinct, heterogeneous polysaccharide-protein complexes isolated from cultivated mycelia of the Basidomycetes mushroom, Trametes (formerly Coriolus) versicolor, that have been used in many traditional Asian formulas to prevent or treat various types of cancer as well as to treat or prevent manifestations of enteric inflammation (18-21). Danshen (*Salvia miltiorrhizae*), which historically, is the TCM chosen for treating circulatory disorders, have also been used in treating certain cancers and as chemopreventive agents (22,23). I'm-Yunity-Plus suppressed clonogenicity to a degree comparable to the effects of adding Danshen alone, and both were more active than cells treated with I'm-Yunity. However, extract of Danshen was more active in inhibiting MCF-7 cell growth than I'm-Yunity-Plus. In comparison, I'm-Yunity showed less growth inhibition. Flow cytometric analysis showed that I'm-Yunity-Plus induced partial block of G_1/S transition in MCF-7 cells, whereas Danshen slowed down cell progression from G_1/S into G_2/M phases of the cell cycle. I'm-Yunity, however, only actively induced apoptosis in MCF-7 cells without affecting cell cycle progression. In addition, only cells treated with Danshen resulted in a pronounced reduction in the expression of Rb, cyclin D1, and p53, as well as diminished levels of p65 and p50 forms of NF-κB. The pronounced suppressive effects of Danshen on expression of the aforementioned genes were largely attenuated in cells identically treated with I'm-Yunity-Plus suggesting that there must have been interactions between ingredients in Danshen with those in I'm-Yunity, the molecular outcome of which is the neutralization of the gene suppressive effects of Danshen. Support for such interactions was also evident using cDNA array analyses targeting tumor metastasis and BCa/ER signaling genes.

Taken together, our results are consistent with the interpretation that interaction exists between Danshen and I'm-Yunity and that I'm-Yunity-Plus may be considered as complementary therapy for patients diagnosed with BCa, particularly individuals with ER^+ status.

Materials and methods

Cell cultures. The breast adenocarcinoma cell line MCF-7 was purchased from American Type Culture Conditions (ATCC, Rockville, MD). Cells were maintained in Eagle's minimum essential medium supplemented with 2 mM glutamine and Earle's BSS adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate and supplemented with 0.01 mg/ml bovine insulin and 10% fetal bovine serum, as described (6). Cells were seeded at a density of $5x10^4$ cells/ml and passaged by washing the mono-layers with phosphate-buffered saline (PBS) followed by a brief incubation with 0.25% trypsin or trypsin/EDTA. The washed cells were isolated by centrifugation and resuspended in culture medium for plating or counting.

Preparation of ethanolic extracts of I'm-Yunity, Danshen and I'm-Yunity-Plus. I'm-Yunity, Danshen, and I'm-Yunity-Plus were provided as capsules or powder by Integrative Chinese Medicine Holdings Ltd. (TST East Kowloon, Hong Kong) and stored at 4˚C. To prepare 70% ethanolic extracts, contents of each capsule of I'm-Yunity and I'm-Yunity-Plus were suspended in 70% ethanol (340 gram/3.3 ml), followed by stirring the suspension at room temperature with intermittent mixing at 150 rpm for 60 min. Since each capsule of I'm-Yunity-Plus contained 110 mg of Danshen, extracts of Danshen were prepared by suspending 110 mg of Danshen in 3.3 ml of 70% ethanol. For each preparation, the insoluble material was removed by centrifugation in a micro-centrifuge and the soluble supernatant was sterilized by passing through a 0.22 μM filter and kept in aliquots at 4˚C. Before use, the stock was further diluted in tissue culture media to give the final indicated concentrations.

Effect of ethanol extracts of I'm-Yunity, I'm-Yunity-plus and Danshen on colony formation and cell proliferation in MCF-7 cells. Clonogenicity was assayed as described (6,12). MCF-7 cells at 200 cells/ml RPMI-1640 and 10% FBS were dispensed into individual wells of a 6-well tissue culture dish. Cultures received various doses of ethanol extracts of I'm-Yunity, I'm-Yunity-Plus or Danshen. Control cultures received equivalent amounts of 70% ethanol. After 14 days in culture the cells were fixed and stained with 0.1% crystal violet to visualize colonies for counting. The experiments were performed in duplicate or triplicate.

To determine effects of ethanolic extracts of I'm-Yunity, I'm-Yunity-Plus or Danshen on inhibition of cell growth, confluent MCF-7 cells were harvested by trypsinization and adjusted to a density of $5x10^4$ cells/ml. For each treatment,

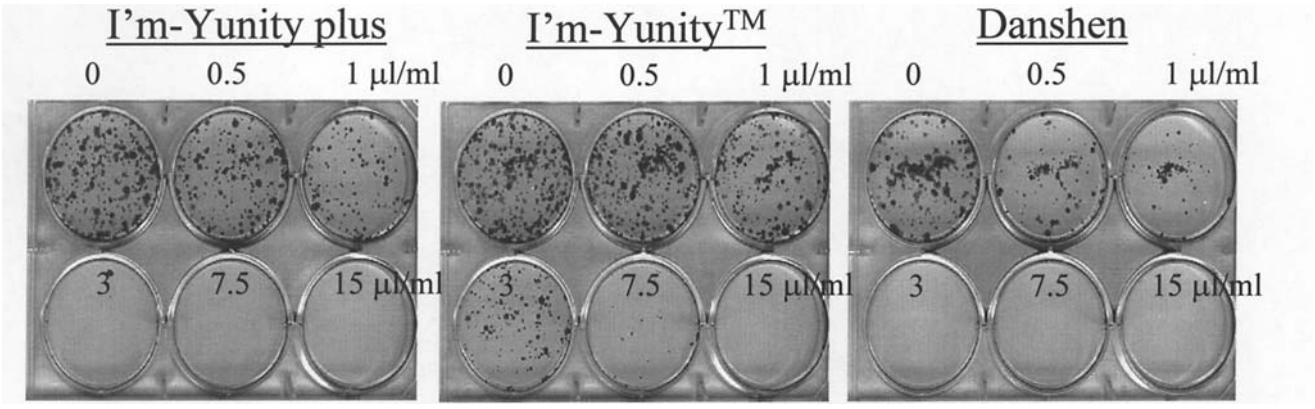


Figure 1. Effects of ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, or Danshen on clonogenicity in MCF-7 cells. Clonogenicity was assayed as detailed in Materials and methods.

10 ml of cells were placed in T-75 flasks, together with 7.5 μl/ml of 70% ethanol extracts of I'm-Yunity, I'm-Yunity-plus, Danshen, or the carrier solvent. Cells were harvested by trypsinization on days 1-3. Cell number in control and treated cells was counted using hemocytometer and cell viability was determined by trypan blue dye exclusion (24-26).

Effect of I'm-Yunity, Danshen and I'm-Yunity-Plus on induction of apoptosis and cell cycle progression. Cell cycle phase distribution was analyzed by flow cytometry. Cultures seeded at a density of $5x10^4$ cells/ml were incubated with 7.5 μl/ml of 70% ethanol extracts of I'm-Yunity, I'm-Yunity-plus, Danshen, or the carrier solvent for 3 days and harvested. Cells were washed once with PBS and stained with 1.0 μg/ml 4,6-diamidion-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) in a solution containing 100 mM NaCl, 2 mM $MgCl_2$ and 0.1% Triton X-100, pH 6.8 as described (27-29). The DNA-specific DAPI fluorescence was excited with UV light and collected with appropriate filters in an ICP-22 flow cytometer (Ortho Diagnostic, Westwood, MA). The cell cycle distribution and percentage of apoptotic cells were analyzed by deconvoluting the DNA content frequency histograms with the use of CellFit software (Phoenix Flow, San Diego, CA), as detailed previously (30,31).

cDNA array analysis. Total RNA was isolated from day 2 control and MCF-7 cells treated respectively with 7.5 μl/ml ethanol extracts of I'm-Yunity, I'm-Yunity-plus, Danshen, or the carrier solvent. RNA (4 μg) was used as the template for biotinylated cDNA synthesis. Hybridization of biotinylated cDNA to immobilized gene-specific cDNAs and detection of hybridization signals by chemiluminescence were according to the manufacturer's protocol (Superarray, Bethesda, MD). Briefly, the steps involved were: 1) prehybridization of membranes in solution containing 100 μg/ml heat-denatured sheared salmon sperm DNA, 2 h, 60˚C; 2) overnight hybridization of membrane with the denatured cDNA probe, with continuous shaking at 10 rpm/min, 60˚C; 3) washing of membrane with pre-warmed 2X SSC and 1% SDS, followed by second washing with 0.1X SSC and 0.5% SDS; 4) blocking of membrane, 40 min using blocking solution Q provided by the manufacturer; 5) incubation of membrane with 1:7500 AP-streptavidin, 10 min, room temperature; 6) incubation of washed and rinsed membrane with 1.0 ml CDP-Star chemiluminescent substrate, followed by exposure to X-ray film. Routinely, multiple exposures were obtained to have more accurate and quantitative determination of the data. GEArray analyzed software was used for the data analysis.

Protein extraction and Western blot analysis. Control MCF-7 cells and cells treated with 7.5 μl/ml ethanol extracts of I'm-Yunity, I'm-Yunity-plus, Danshen, or the carrier solvent were rinsed with ice-cold PBS and suspended in buffer (50 μl/10^6 cells) containing 10 mM Hepes, pH 7.5, 90 mM KCl, 1.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 0.5% NP40 and 5% glycerol supplemented with 0.5 mM PMSF, 10 μg/ml each of aprotinin, pepstatin, leupeptin, and lysed by 3 freeze/thaw cycles (6,26,30,31). The extracts were centrifuged and the clear supernatants were stored in aliquots at -70˚C. Protein concentrations were measured with protein assay reagent (Pierce Chem. Co., Rockford, IL). For Western blot analysis, 10 μg proteins were boiled for 5 min in Laemmli buffer and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gels were transferred to nitrocellulose membranes by a semi-dry transfer method. After blocking with buffer containing 5% low-fat milk, the membranes were probed for the expression of Rb, cyclins D1, p53, p21, NF-κB p65 and p50, and ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA). All antibodies used in the experiments were diluted at 1:1000. Specific immunoreactivity was demonstrated by enhanced chemiluminescence (ECL) or color reaction using procedures detailed in the manufacturer's protocol (Kirkegared & Perry Laboratories, Gaithersburg, MD).

Results

Effects of ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen on colony formation in MCF-7 cells. We first determined the effects of I'm-Yunity, I'm-Yunity-plus, and Danshen on colony formation, also referred to as clonogenicity, in MCF-7 cellls. This assay measures the ability of tumor cells to grow and form foci in a manner unrestricted by growth contact inhibition as is characteristically found in normal, untransformed cells. As such, clonogenicity provides an indirect assessment of the propensity of tumor cells to undergo neoplastic transformation. To measure clonogenicity, MCF-7 cells at a given cell density were plated onto multiple well tissue culture dishes, with and without addition of varying

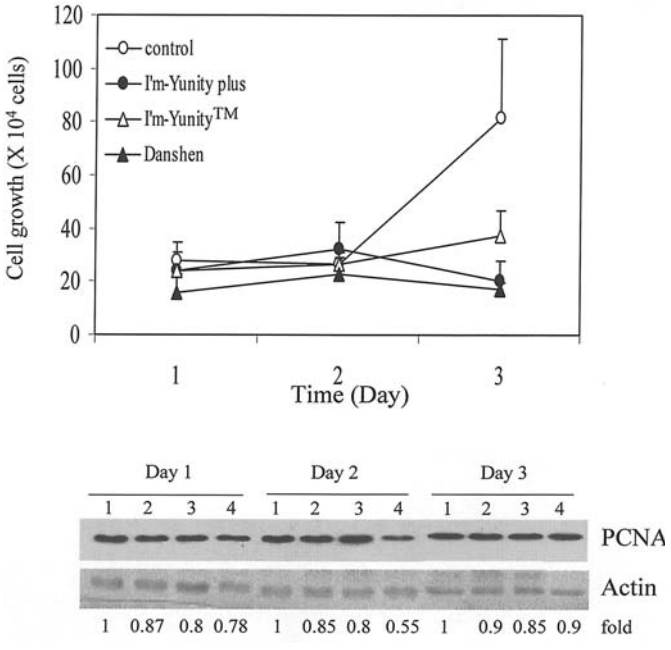


Figure 2. Time-dependent growth inhibition and PCNA expression in control and MCF-7 cells treated with 7.5 μl/ml ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, or Danshen.

doses of 70% ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen. Control and treated cells were maintained in culture for an additional 14 days to allow formation of colonies. Size and number of colonies were visually inspected by fixing and staining in 0.1% crystal violet. Fig. 1 shows that clonogenicity of MCF-7 cells was significantly reduced by incubation with ethanol extracts of I'm-Yunity-plus and Danshen, with both showing a pronounced inhibition that was clearly evident at ≥1-3 μl. Notably, not only did Danshen reduced the number of colonies formed to a degree similar to treatment with I'm-Yunity-plus, it also visibly modulated the size of colonies formed, decreasing the sizes of some while stimulating those of others. Comparatively, 70% ethanolic extracts of I'm-Yunity were significantly less active in this assay, both with respect to the number and size of colonies formed as evident by the observation that inhibition became visually apparent only when the doses reached 7.5-15.0 μl (Fig. 1).

Effects of ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen on growth of MCF-7 cells. We next tested the effects of I'm-Yunity, I'm-Yunity-plus, and Danshen on growth of MCF-7 cells. Results in Fig. 2A showed effects of 7.5 μl/ml of ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen. Significant reduction in cell growth was observed in cells treated with I'm-Yunity-plus and Danshen, while the inhibitory effects of I'm-Yunity were noticeably less prominent (panel A, Fig. 2). The suppression of cell proliferation by Danshen was accompanied by a transient reduction in expression of PCNA on day 1, which became progressively more pronounced on day 2 and returned to control levels on day 3 of treatment. Neither I'm-Yunity or I'm-Yunity-plus suppressed PCNA expression.

Treatment (3 days)	Dose (μl/ml)	Cell cycle distribution (%)			
		G1	S	G2/M	Apoptosis
control	0	48.2	36.8	15.1	
I'm-Yunity plus	7.5	57.2	29.8	13.0	5.4
I'm-Yunity™	7.5	50.9	34.7	14.5	21.2
Danshen	7.5	57.4	34.4	8.3	

Figure 3. Cellular DNA content frequency histograms of MCF-7 cells showing the cell cycle phase distribution and apoptosis following a 3-day treatment with 7.5 μl/ml ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, or Danshen. Flow cytometric analysis was performed as described in Materials and methods. Proportion of cells with fractional DNA content (sub-G_1 cell population) was used to calculate induction of apoptosis resulting from treatment.

Effects of ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen on cell cycle progression in MCF-7 cells. To explore the underlying basis for the antiproliferative activities of *I'm-Yunity, I'm-Yunity-plus*, and *Danshen*, cell cycle analyses were performed in MCF-7 cells, following a 3-day treatment with 7.5 μl/ml of ethanolic extracts. Cell cycle phase distribution was analyzed by flow cytometry. Control and treated cultures were harvested, washed with PBS and stained with 1.0 μg/ml DAPI, and analyzed as detailed in Materials and methods (27,31). Results in Fig. 3 indicate that ethanol extracts of I'm-Yunity-Plus induced partial block of G_1/S transition in MCF-7 cells, whereas Danshen slowed down cell progression from G_1/S into G_2/M phases of the cell cycle. By comparison, I'm-Yunity had virtually no effect on cell cycle progression but actively induced apoptosis in MCF-7 cells.

Modulation of cell growth regulatory protein expression by of ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen in MCF-7 cells. Further evidence that these three herbal extracts elicited different cell cycle effects in MCF-7 cells came from analysis of changes in expression of proteins with key role in regulating cell cycle progression. For instance, MCF-7 cells treated with Danshen alone showed greatly suppressed expression of cyclin D1, whereas identical treatment with I'm-Yunity and I'm-Yunity-Plus had virtually no effect (Fig. 4). We also analyzed for changes in the retinoblastoma tumor suppressor protein Rb, whose expression and state of phosphorylation play a pivotal role in the control of G_1/S cell cycle checkpoint (28). Western blot analysis demonstrated that treatment with 7.5 μl/ml ethanolic extract of Danshen significantly reduced the overall Rb levels, in MCF-7 cells (Fig. 4). Similarly, expression of p53 was down-regulated by ethanol extracts of Danshen but were unaffected by treatment with I'm-Yunity and I'm-Yunity-Plus.

The changes in cyclin D1 may explain in part the observed effect of Danshen on suppression of S/G_1 in MCF-7 cells but cannot easily support the apoptosis inducing effects of I'm-Yunity in MCF-7 cells. Accordingly, we measured changes in the steady state level of transcription factor NF-κB, known to be functionally associated with cell survival (32,33). However, once again, only treatment with ethanolic extract of Danshen elicited a pronounced decrease in p65 and p50 forms of NF-κB in MCF-7 cells (Fig. 5).

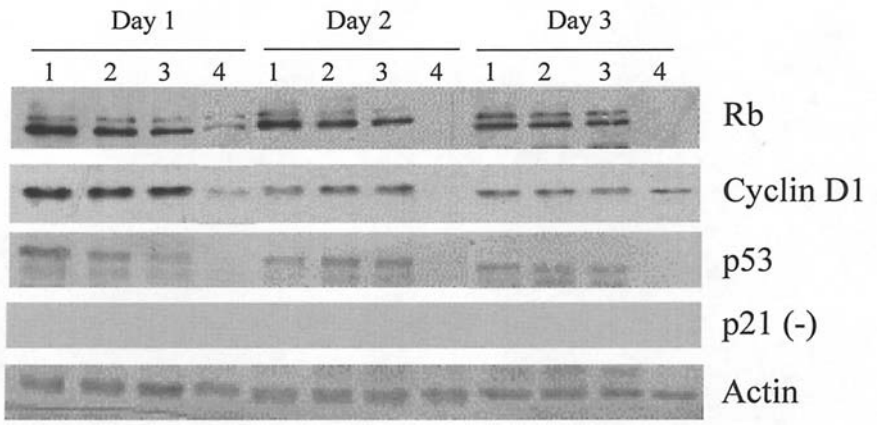


Figure 4. Expression of Rb, cyclin D1, p53 and p21 expression was investigated by immunoblot analysis as detailed in Materials and methods in MCF-7 cells treated for 1-3 days with 7.5 μl/ml ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, or Danshen. A significant down-regulation on Rb, cyclin D1 and p53 was found in cells treated with Danshen.

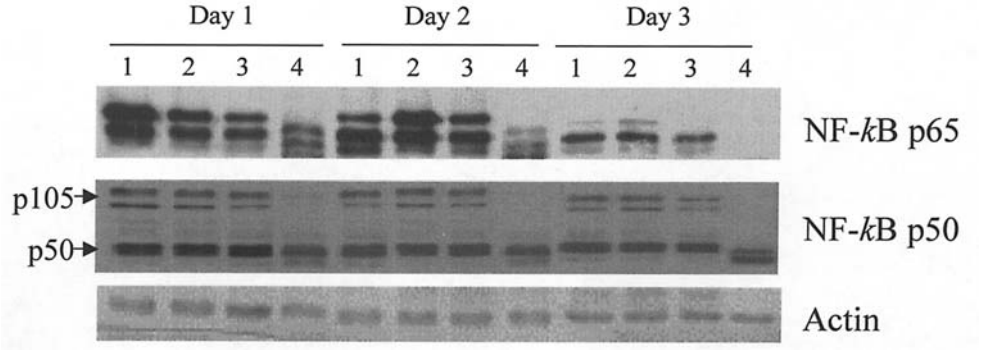
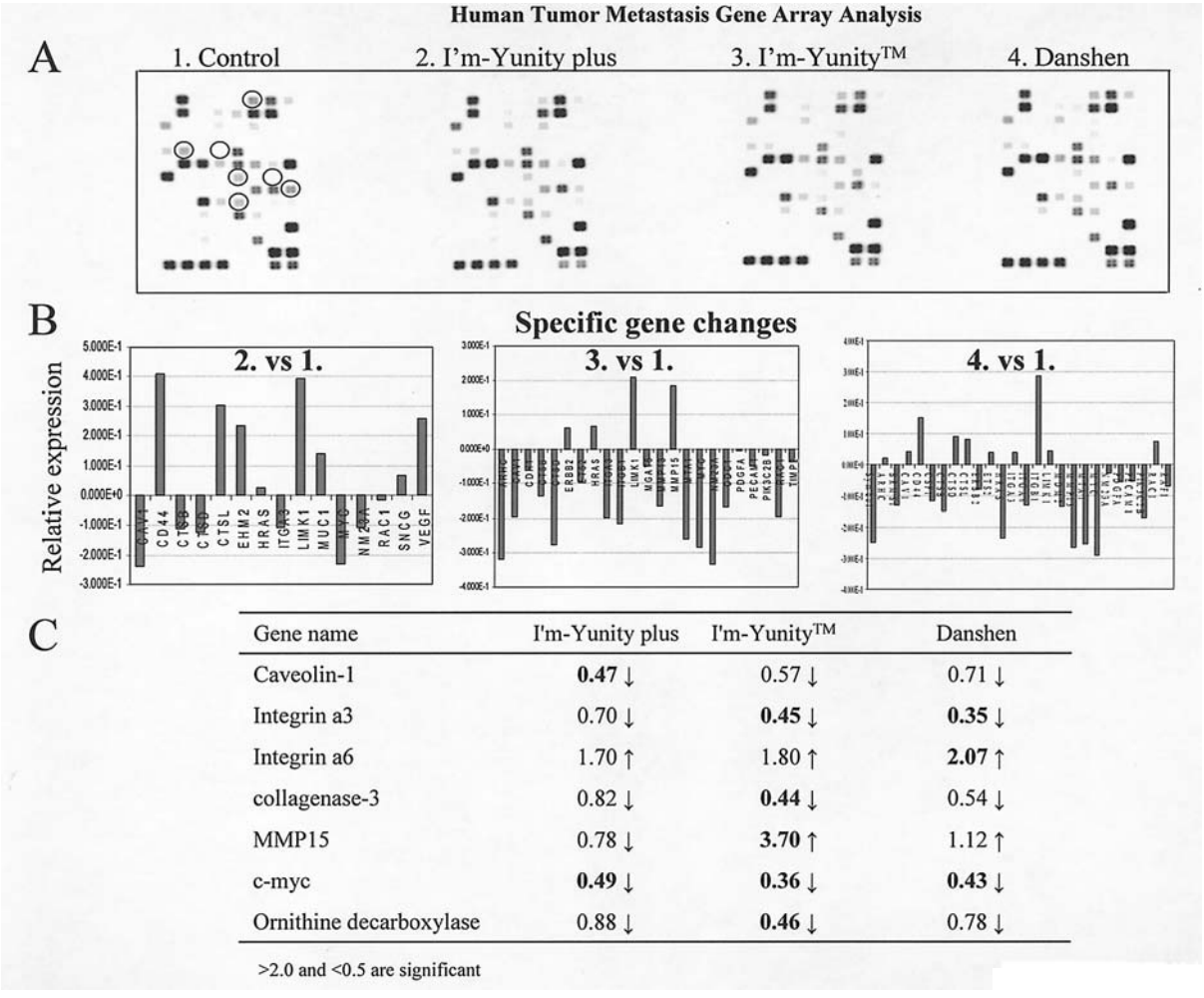


Figure 5. Expression of p65 and p50 forms of NFκB was investigated by immunoblot analysis as detailed in Materials and methods in MCF-7 cells treated for 1-3 days with 7.5 μl/ml ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, or Danshen. A significant down-regulation on NFκB was found in cells treated with Danshen.

Effects of ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen on expression of tumor metastasis gene expression using cDNA array analysis. Human tumor metastasis cDNA array was used to detect changes in expression of a panel of cycle regulatory genes, in response to treatment with ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen. This array contains genes that encode for several classes of protein factors including growth factors and their receptors, ECM components, proteases and their inhibitors, oncogenes and suppressors, and other related genes. This array can provide some insights into the molecular mechanism of metastasis or determine how a tumor metastasize. Through a side-by-side hybridization using experimental RNA samples and the arrays and reagents provided in the kit, it would be possible to simultaneously determine the expression profile of these genes involved in metastasis.

In these experiments, cells treated for 2 days were used in the analysis. Only integrin a6 showed a consistent up-regulation in response to treatment by all three herbal extracts. Expression of MMP15 was significantly increased by treatment with I'm-Yunity and was marginally increased by treatment with Danshen. Interestingly, MMP15 was significantly suppressed by I'm-Yunity-Plus. In addition, a number of other genes, notably, caveolin, c-myc and ornitheine decarboxylase, were also substantially suppressed by all three herbal extracts (Fig. 6).

Effects of ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen on expression of breast cancer and estrogen receptor signaling genes using cDNA array analysis. We further analyzed gene responses using human breast cancer/estrogen receptor signaling cDNA arrays. The GEArray Q series human breast cancer/estrogen receptor signaling gene array is designed for the investigation of breast cancer-related gene regulation and estrogen receptor-dependent signal transduction. Although the estrogen receptor plays an important role in the pathogenesis of many breast cancers, a number of additional estrogen-independent signaling pathways are also associated with the disease. Many genes are known to be preferentially expressed in breast cancer cells and thus serve as useful markers of the disease. The expression of other genes defines the prognosis of breast cancer, while the expression of still other genes is associated with the response of cancer cells to chemotherapy. All of these genes as well as those involved in the estrogen-signaling pathway are included on this array (Fig. 7).



Gene name	I'm-Yunity plus	I'm-Yunity™	Danshen
Caveolin-1	**0.47** ↓	0.57 ↓	0.71 ↓
Integrin a3	0.70 ↓	**0.45** ↓	**0.35** ↓
Integrin a6	1.70 ↑	1.80 ↑	**2.07** ↑
collagenase-3	0.82 ↓	**0.44** ↓	0.54 ↓
MMP15	0.78 ↓	**3.70** ↑	1.12 ↑
c-myc	**0.49** ↓	**0.36** ↓	**0.43** ↓
Ornithine decarboxylase	0.88 ↓	**0.46** ↓	0.78 ↓

>2.0 and <0.5 are significant

Figure 6. Identity and cellular role of human metastasis genes regulated by a 2-day treatment with 5.5 μl/ml ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, or Danshen. The GEArray Q series human tumor metastasis gene array is designed to represent 96 genes known to be involved in metastasis. Quantitative analysis of genes circled in panel A appear in panels B and C, respectively.

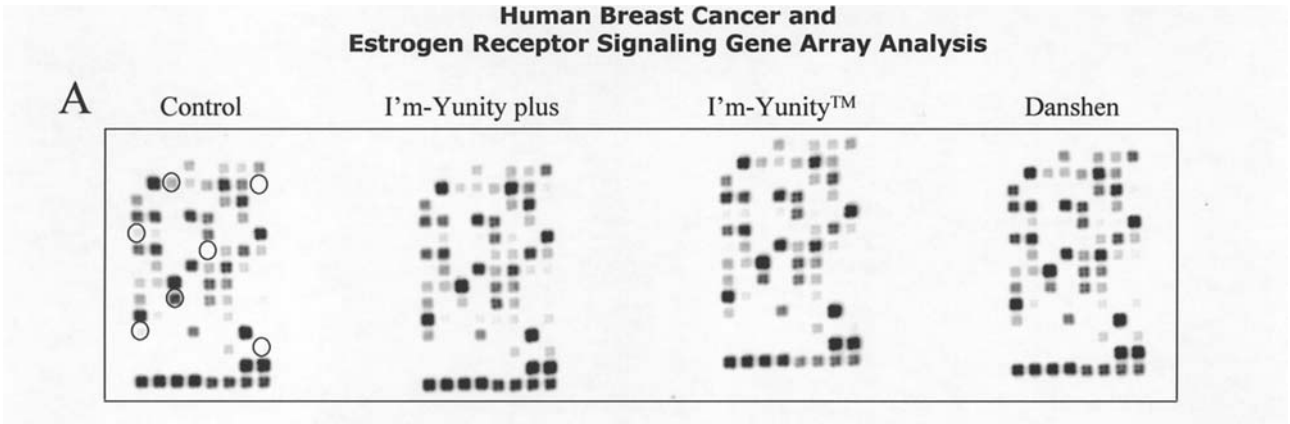
Again, cells treated for 2 days with ethanol extracts of I'm-Yunity, I'm-Yunity-plus, and Danshen were used in the analysis. These studies revealed a significant up-regulation of p21. Other genes whose expression increased included IL-6 and VEGF. Suppression of PR and FGF1 was also found.

Discussion

I'm-Yunity and Danshen are popular dietary supplement in the Orient, which show beneficial effects with little toxicity (34). The main active components of I'm-Yunity is a polysaccharo-peptide (18-20). In the case of Danshen, several bioactive compounds have been identified (35-37). Since these bioactive agents have substantially different biochemical characteristics, we reasoned that combination of I'm-Yunity and Danshen may interact in ways that provide evidence for biological synergy. This possibility appears to be validated by our studies using human MCF-7 cells.

We observed that proliferation in MCF-7 cells was inhibited to a comparable degree by I'm-Yunity-plus and Danshen, and to a lesser degree, also by I'm-Yunity (Fig. 2A). Clonogenicity assay using these cells, however, showed Danshen to be more effective than I'm-Yunityplus and I'm-Yunity (Fig. 1). Flow cytometric analysis revealed that 7.5 μl/ml Danshen induced G_1/S arrest while I'm-Yunity-plus elicited a prolongation of G_1/S transition into G_2/M phase of the cell cycle (Fig. 3). Little apoptosis was shown in MCF-7 cells treated with I'm-Yunity-plus or Danshen, which was strikingly different from the significant induction of apoptosis in cells treated with I'm-Yunity (Fig. 3). To investigate the biochemical basis for the observed flow cytometric results, we analyzed changes in cell cycle regulatory proteins. Only Danshen-treated MCF-7 cells showed diminished expression of cyclin D1, Rb and p53. Similarly, p65 or p50 forms of NFκB were affected by Danshen in MCF-7 cells (Fig. 5). These results contrasted with little to no observed changes in cyclin D1, Rb and p53, or either form of NFκB in MCF-7 cells treated with either I'm-Yunity or I'm-Yunityplus (Figs. 4 and 5).

Control of tumor metastasis and estrogen receptor signaling by I'm-Yunity, I'm-Yunity-plus, and Danshen: analysis using cDNA arrays. To further investigate that synergy could arise from combining Danshen with I'm-Yunity, we resorted to comparison of the expression profile of gene sets that either regulate or are involved in tumor metastasis and estrogen receptor signaling in MCF-7 cells. To assess the relative



B

Gene name	I'm-Yunity plus	I'm-Yunity	Danshen
Cyclin D1	**0.44** ↓	1.02 ↑	0.86 ↓
p21/Waf1/CIP1	**3.76** ↑	**2.06** ↑	**2.15** ↑
FGF1	**0.36** ↓	0.81 ↓	0.54 ↓
IL6	**16.01** ↑	**2.75** ↑	**6.92** ↑
PR	**0.27** ↓	0.95 ↓	0.69 ↓
CD98	**3.60** ↑	**2.39** ↑	**3.27** ↑
VEGF	**2.81** ↑	1.29 ↑	**2.29** ↑

>2.0 and <0.5 are significant

Figure 7. Identity and cellular role of breast cancer/estrogen receptor signaling genes regulated by a 2-day treatment with 5.5 μl/ml ethanolic extracts of I'm-Yunity, I'm-Yunity-plus, or Danshen. The GEArray Q series human breast cancer/estrogen receptor signaling gene array is designed for the investigation of breast cancer-related gene regulation and estrogen receptor-dependent signal transduction. Quantitative analysis of genes circled in panel A appear in panel B.

response of functionally related gene sets quickly and accurately, a cDNA array analysis was performed. Unlike classic RNA methodologies such as Northern blot analysis, studying one gene at a time, low-density arrays were selected for ease of operation and management of data; comparison of the differential hybridization outputs between control and treated MCF-7 cells enabled systematic and reliable comparison of relative levels of many mRNA transcripts simultaneously in a single experiment. Results derived from computer analysis provided semi-quantitative information that may provide clues to an understanding of complex pathways and their interactions in gene regulation that underlie hormone-dependent breast carcinogenesis and that may be targeted by I'm-Yunity, I'm-Yunity-plus, and Danshen.

By comparing the expression profile after 2 day of treatment with I'm-Yunity, I'm-Yunity-plus, and Danshen, we found that p21 was the most highly induced gene in treated MCF-7 cells. The p21 is a potent mitotic inhibitor, which inhibits cdc2 expression. Additionally, the p21 can also inhibit Rb phosphorylation by binding to cdk to effectively block the catalytic activities of cdk-cyclin complexes, e.g., cyclins A/E-cdk2, and cyclin D1/D2-cdk4. Moreover, the highly induced expression of p21 following I'm-Yunity, I'm-Yunity-plus, and Danshen treatment would ensure the growth arrest at G_1. Our array analysis revealed this to be an unreported target of I'm-Yunity, I'm-Yunity-plus, and Danshen.

The use of gene array analysis should provide the broadest scientific framework relevant to the anti-BCa properties of I'm-Yunity, I'm-Yunity-plus, and Danshen. Furthermore, since substantial variation in efficacy exists between different batches of I'm-Yunity, I'm-Yunity-plus, and Danshen, and methods for their standardization are as yet unavailable, the gene profile approach could also subserve as a reproducible, sensitive, and easily adaptable laboratory indicator confirming/refuting the lot-to-lot consistency status of I'm-Yunity, I'm-Yunity-plus, and Danshen, and may be used to guide the development of I'm-Yunity, I'm-Yunity-plus, and Danshen with more potent biological properties.

In summary, our results are consistent with the interpretation that interaction exists between Danshen and I'm-Yunity and that I'm-Yunity-Plus may be useful in the treatment of BCa, particularly in patients with ER^+ status.

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