Identification of plant extracts sensitizing breast cancer cells to TRAIL

SHERIF ABDELHAMED[1], SATORU YOKOYAMA[1], LIA HAFIYANI[1], SURYA K. KALAUNI[2], YOSHIHIRO HAYAKAWA[1], SURESH AWALE[1,3] and IKUO SAIKI[1]

[1]Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, Toyama, Japan; [2]Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal; [3]Frontier Research Core for Life Sciences, University of Toyama, Toyama, Japan

Received November 19, 2012; Accepted January 7, 2013

DOI: 10.3892/or.2013.2293

Abstract. Triple-negative breast cancer (TNBC) is an aggressive heterogeneous cancer subgroup with a higher rate of distant recurrence and a poorer prognosis compared to other subgroups. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an attractive molecule that induces cell death in various tumor cells without causing cytotoxicity to normal cells; however, primary or acquired resistance to TRAIL often limits its efficacy in cancer patients. To develop combination therapies to improve TRAIL efficacy and/or to overcome the resistant mechanism, we screened 138 medicinal plant extracts against TRAIL-sensitive and -insensitive TNBC cell lines, MDA-MB-231 and MDA-MB-468. Among them, 5 plant extracts, *Uvaria dac, Artemisia vulgaris, Cortia depressa, Dichasia bengalensis* and *Cinnamomum obtusifolium* did not cause apparent cytotoxicity (<20%) as a single regimen, but showed significant synergistic effects in combination with TRAIL against both cell lines. Moreover, *Uvaria dac, Artemisia vulgaris* and *Cinnamomum obtusifolium* were found to suppress the phosphorylation of p65 that is involved in TRAIL-resistant mechanisms. These observations suggest that the identified plant extracts in combination with TRAIL could lead to potential therapeutic benefits for cancer patients in the clinical setting.

Introduction

Breast cancer is one of the most frequently diagnosed types of cancer and is also the leading cause of cancer-related mortality among women, representing 23% of total cancer cases and 14% of cancer-related mortality (1). Breast cancer is classified into different categories according to the expression of three receptors, estrogen receptor (ER), progesterone receptor (PR), and HER2/Neu (2). A triple-negative breast cancer (TNBC), which is ER-negative, PR-negative, and HER2-negative, is one of the most aggressive forms that accounts for 15-25% of all breast cancer cases and is associated with a poor prognosis and unresponsiveness to the usual endocrine therapies (3-5).

The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that can initiate apoptosis via the activation of death receptor 4 (DR4) and death receptor 5 (DR5). As TRAIL can selectively induce apoptosis in cancer cells, including breast cancer cells, without causing toxicity to normal cells (6-8), it could be a safe medication for cancer patients; however, primary or acquired resistance to TRAIL is often observed which may limit its efficacy in cancer patients (6). Therefore, increasing TRAIL efficacy by combining with natural medicines or other chemotherapeutic agents is an important strategy in the treatment of breast cancer in the clinical setting. We previously identified that vanillin, an active constituent of vanilla, enhances TRAIL-induced apoptosis in cancer cells via suppression of NF-κB (9). This finding prompted us to search for other natural products that can sensitize cancer cells to TRAIL or that can overcome the TRAIL-resistant mechanism.

In the present study, we screened 138 medicinal plant extracts in TNBC cells to identify candidates that are not cytotoxic as a single agent but that can increase TRAIL-induced cytotoxicity synergistically. Among the tested extracts, 5 extracts *(Uvaria dac, Artemisia vulgaris, Cortia depressa, Dichasia bengalensis and Cinnamomum obtusifolium)* were found to activate TRAIL effects in TRAIL-sensitive TNBC MDA-MB-231 cells and to overcome unresponsiveness in TRAIL-insensitive TNBC MDA-MB-468 cells. We herein report the TRAIL modulatory activity by these identified extracts and their mechanisms of action.

Materials and methods

Plant extracts and reagents. Plant extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1,000 μg/ml and these stock solutions were stored at -20˚C. Recombinant human TRAIL was purchased from PeproTech (Peprotech, London, UK). The plants used in this study are listed in Table I.

Correspondence to: Professor Ikuo Saiki, Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
E-mail: byosei@inm.u-toyama.ac.jp

Key words: TRAIL, breast cancer cell, medicinal plant extract

Cell culture. MDA-MB-231, MDA-MB-468, MDA-MB-453 and MCF-7 cells (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2/95% air.

Cell viability assay. Viability of cells following treatment was determined using the WST-1 Cell Counting kit (Wako Pure Chemical Industries, Osaka, Japan) as previously described (10). Briefly, cells were seeded into 96-well plates ($7x10^3$/80 μl/well). After 24 h, 10 μl medium containing each plant extract was added and cells were incubated for an additional 30 min. After the addition of TRAIL (50 ng/ml), cells were incubated for 24 h, and 10 μl WST-1 solution was added to each well and incubated at 37˚C for 1 h. The absorbance at 450 nm was measured. Relative cell viability was calculated by the formula: Relative cell viability = [average absorbance of experimental wells/average absorbance of control wells]. Synergistic effects were determined when cell viability treated with both TRAIL and plant extract (V_{comb}) was less than predicted additive effects [= cell viability of TRAIL alone (V_{TRAIL}) x cell viability of plant extract alone (V_{ext})]. The synergy index was calculated by $V_{com}/(V_{TRAIL} \times V_{ext})$.

Western blot analysis. Western blot analysis was performed as previously described (11). Cells were seeded and incubated overnight in a 60-mm culture dish ($0.25x10^6$ cells/ml). Following treatment, whole cell lysates were collected in lysis buffer [25 mM HEPES pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 10 mg/ml aprotinin and 10 mg/ml leupeptin]. Equal amounts of protein were resolved by electrophoresis on acrylamide gels. Antibodies against caspase-3, PARP, XIAP, MCL-1 and phosphorylated p65 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β-actin and p65 were purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA, USA).

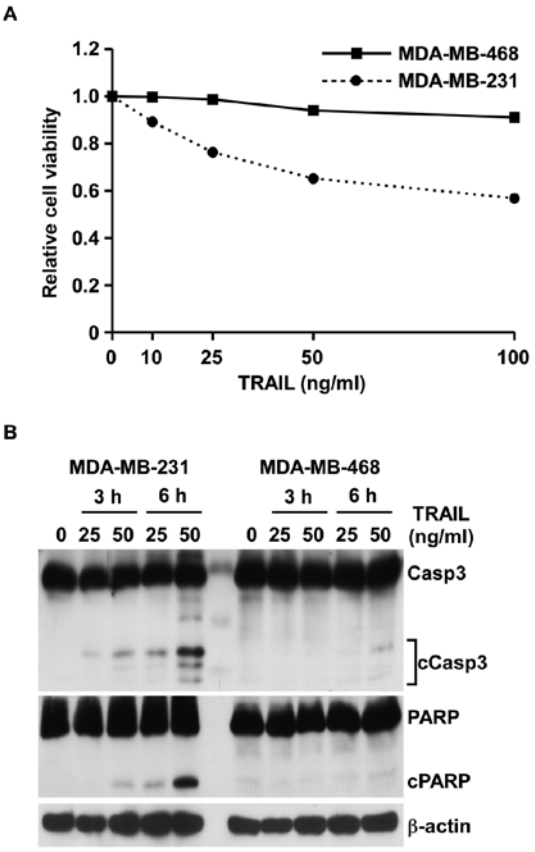
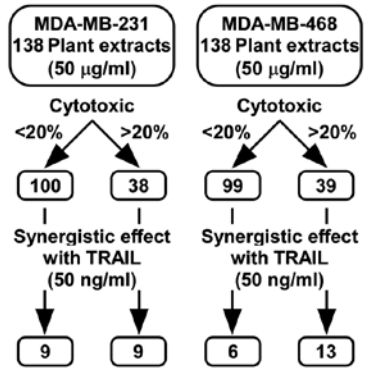


Figure 1. TRAIL sensitivity in breast cancer cell lines. (A) Cell viability assay in MDA-MB-231 and MDA-MB-468 cells. Cells were treated with various concentrations of TRAIL for 24 h. Results are normalized to the vehicle control in each cell line. (B) Expression of cleaved caspase-3 and cleaved PARP in MDA-MB-231 and MDA-MB-468 cells detected by western blot analysis. Cells were treated with the indicated concentrations of TRAIL for the indicated times.

Results

TRAIL sensitivity in breast cancer cell lines. Since each cell line shows various sensitivities to TRAIL due to different expression levels of BAX, MCL-1, IAPs and others (12-17), we initially tried four breast cancer cell lines, MDA-MB-231, -468, -453 and MCF-7, to distinguish TRAIL-sensitive from -insensitive cells. Of note, the cell viability assay showed that only MDA-MB-231 cells were sensitive to TRAIL and the others were insensitive (Fig. 1A and data not shown). Both MDA-MB-231 and MDA-MB-468 are triple-negative (ER^-, PR^- and $HER2^-$) breast cancer cells, and are reported to be the most severe forms of breast cancer (3-5); therefore, we focused on these two cell lines in the present study. Furthermore, cleaved PARP and cleaved caspase-3, which are known as apoptotic markers, were also observed in MDA-MB-231 cells with TRAIL in a time- and concentration-dependent manner, but not in MDA-MB-468 cells (Fig. 1B). This showed that MDA-MB-231 cells are sensitive to TRAIL and MDA-MB-468 cells are insensitive, and that the cell death induced by TRAIL might be due to apoptosis.

Classification of the plant extracts according to their cytotoxic and/or synergistic effects. Subsequently, we identified the plant extracts that showed less cytotoxicity as a single agent, but that enhanced TRAIL effects in TRAIL-sensitive cells or that overcame unresponsiveness in TRAIL-insensitive cells. One hundred and thirty-eight plant extracts with or without TRAIL were screened by cell viability assay (Fig. 2 and Table I). Two criteria were used to classify the plant extracts; first, we determined whether cell viability treated with the plant extracts alone (V_{ext}) was >0.8 (>80% cell survival) at the maximum tested concentration of 50 μg/ml. Second, we checked whether the combination of plant extract with TRAIL showed a synergistic effect, indicating that the detected cell viability with both TRAIL and plant extract (V_{comb}), is less than its predicted additive effect [= cell viability with TRAIL alone (V_{TRAIL}) x V_{ext}] and the synergy index [= $V_{com}/(V_{TRAIL} \times V_{ext})$] is also <0.8. In TRAIL-sensitive MDA-MB-231 cells, 100 extracts showed low cytotoxicity (V_{ext} >0.8). Among them, 9 extracts *(C. fructus, C. obtusifolium, C. japonica, U. dac, A. vulgaris, C. depressa, D. bengalensis, A. venustum* and *P. benghalensis)* showed synergistic effects with TRAIL. In



U. dac	○		○	
A. vulgaris	○		○	
C. depressa	○		○	
D. bengalensis	○		○	
C. obtusifolium	○		○	
C. fructus	○			
C. obtusa		○		○
D. floribumda			○	
C. japonica	○			○
S. dulsis		○		○
E. prostrata		○		
O. valgatum		○		○
A. venustum	○			○
P. benghalensis	○			○
A. mexicana				○
O. indicum		○		○
L. sativus		○		○
C. gigantea		○		○
C. copticum		○		○
W. tinctoria				○
P. chabo		○		○

Figure 2. Screening of 138 medicinal plant extracts and identification of 5 plant extracts sensitizing breast cancer cells to TRAIL. The classifications of 138 plant extracts were determined by the cell viability assay in MDA-MB-231 and MDA-MB-468 cells.

the case of MDA-MB-468 cells, 99 extracts showed low cytotoxicity and 6 extracts *(D. floribumda, C. obtusifolium, U. dac, A. vulgaris, C. depressa* and *D. bengalensis)* showed synergistic effects with TRAIL. As shown in Fig. 2, only 5 extracts met these two criteria in both cell lines, *U. dac*, *A. vulgaris*, *C. depressa*, *D. bengalensis* and *C. obtusifolium*.

Five identified plant extracts induce cell death in a concentration-dependent manner. To check the concentration-dependency of the 5 selected plant extracts, we performed the cell viability assay at various extract concentrations with TRAIL (Fig. 3). These extracts showed synergistic effects with TRAIL in both cell lines in a concentration-dependent manner; therefore, we further examined whether the induced cell death was due to increased apoptosis or due to other types of cell death (Fig. 4). In both cell lines, the cleavage of PARP and caspase-3 was strongly detected when treated with a combination of plant extract and TRAIL compared with TRAIL alone. We then checked the expression and phosphorylation of p65, in addition to the expression of XIAP and MCL-1, which are involved in TRAIL-resistant mechanisms (9,12,14-16).

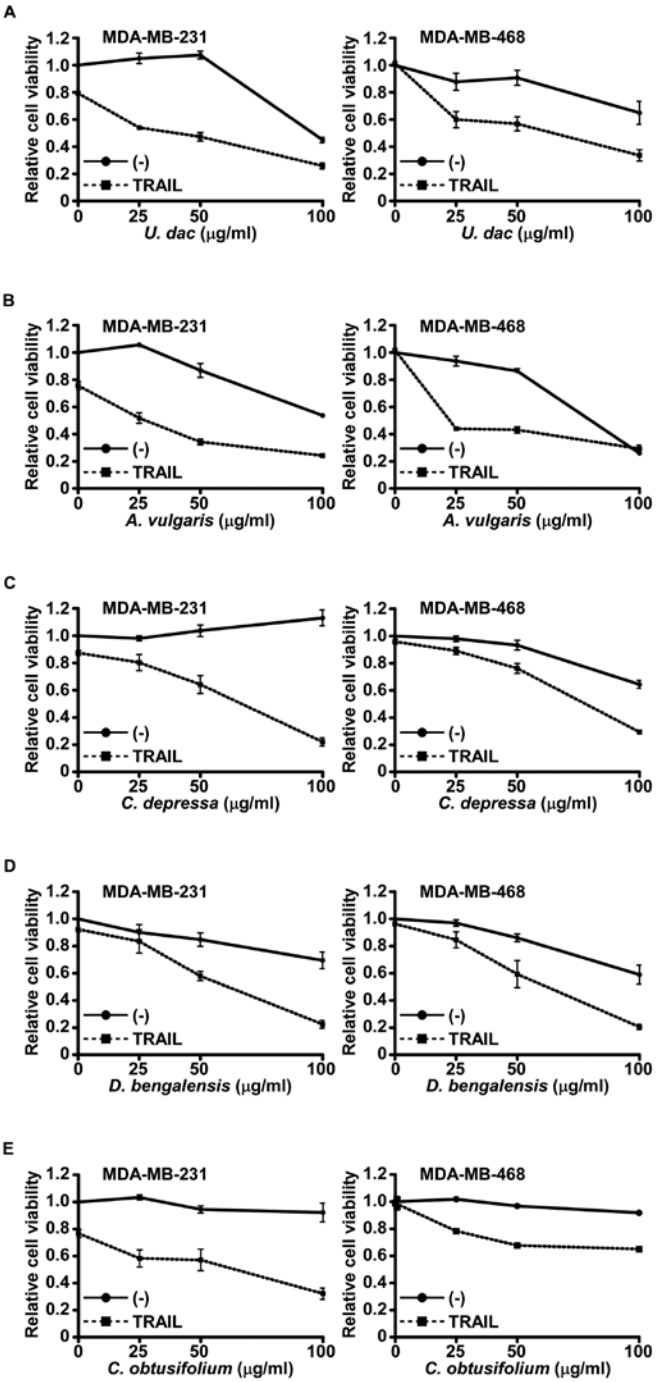


Figure 3. Sensitization of breast cancer cells to TRAIL with the identified plant extracts. (A-E) Cell viability assays in breast cancer cells with TRAIL (50 ng/ml) and 5 plant extracts. Results are normalized to the vehicle control.

Markedly, the three extracts (*U. dac*, *A. vulgaris* and *C. obtusifolium*) showed clear inhibition of the phosphorylation of p65, suggesting that the reduced phosphorylation of p65 could explain the synergistic effect of *U. dac*, *A. vulgaris* and *C. obtusifolium* with TRAIL. On the other hand, the two extracts, *C. depressa* and *D. bengalensis*, that also showed synergistic effects with TRAIL did not show the downregulation of MCL-1, XIAP or phosphorylation of p65, suggesting that these two extracts may cause the synergistic effects with TRAIL by affecting different proteins or pathways.

Table I. Evaluation of 138 plant extracts for their cytotoxicity as a single regimen and/or additive or synergistic effect with TRAIL.

Extract	MDA-MB-231		Predicted additive effect	Synergy Index	MDA-MB-468		Predicted additive effect	Synergy Index
	TRAIL (-)	TRAIL (+)			TRAIL (-)	TRAIL (+)		
	1.00	0.76			1.00	0.97		
Zizyphi Fructus	0.86	0.69	0.66	1.05	1.06	1.00	1.03	0.98
Cinnamomi Cortex	0.88	0.59	0.67	0.88	1.06	1.04	1.03	1.01
Rhei Rhizoma	0.80	0.62	0.61	1.01	0.99	0.98	0.96	1.01
Araliae Cordatae Rhizoma	0.95	0.68	0.72	0.94	1.01	0.95	0.98	0.97
Condonopsitis Radix	0.97	0.72	0.74	0.98	0.96	0.94	0.93	1.01
Ginseng Radix Rubra	1.03	0.80	0.79	1.02	0.95	0.93	0.93	1.00
Salviae miltiorrhizae Radix	1.00	0.75	0.76	0.98	1.01	1.02	0.98	1.04
Armeniacae Semen	0.96	0.72	0.74	0.98	1.04	1.03	1.01	1.02
Eucommiae Cortex	0.89	0.61	0.68	0.89	0.99	0.94	0.96	0.98
Zanthoxyli Fructus	0.99	0.65	0.76	0.86	1.00	0.95	0.97	0.98
Acanthopanax senticosus Harms	1.03	0.70	0.78	0.89	1.03	1.01	1.00	1.01
Morus bombycis Koidz	0.94	0.66	0.71	0.92	1.03	1.03	1.00	1.03
Rehmanniae Radix	1.13	0.88	0.86	1.02	1.04	1.01	1.01	1.01
Platycodi Radix	1.53	1.00	1.17	0.86	1.04	1.07	1.00	1.07
Corni Fructus	1.45	0.81	1.10	0.73	0.97	1.01	0.94	1.07
Sparganii Rhizoma	1.08	0.95	0.83	1.15	0.96	1.02	0.93	1.09
Trichosanthis Radix	1.28	1.03	0.97	1.06	0.93	0.97	0.91	1.07
Lycii Cortex	1.16	1.05	0.89	1.19	0.99	1.00	0.96	1.04
Cannabis Fructus	1.12	0.75	0.86	0.88	1.05	1.10	1.02	1.08
Tribuli Fructus	1.05	0.73	0.80	0.91	1.02	1.11	0.99	1.12
Gentianae macrophyllae Radix	0.97	0.64	0.74	0.86	1.02	1.04	0.99	1.05
Puerariae Radix	1.01	0.79	0.77	1.03	1.08	1.05	1.05	1.00
Zingiberis Rhizoma	0.99	0.71	0.75	0.94	0.94	0.91	0.91	1.00
Alismatis Rhizoma	0.87	0.63	0.66	0.94	0.93	0.92	0.90	1.03
Zingiberis Siccatum Rhizoma	1.10	0.78	0.84	0.93	0.94	0.96	0.91	1.05
Sophorae Radix	1.26	0.93	0.96	0.96	1.00	1.01	0.97	1.05
Rehmannia glutinosa	1.05	0.70	0.80	0.87	1.38	1.43	1.34	1.07
Zedoariae Rhizoma	0.76	0.66	0.58	1.14	0.94	0.97	0.91	1.07
Ephedrae Herba	1.05	0.73	0.80	0.92	0.87	0.87	0.84	1.03
Paeoniae Moutan Cortex	0.93	0.65	0.71	0.91	0.99	0.94	0.96	0.98
Evodiae Fructus	0.93	0.66	0.71	0.93	1.05	1.11	1.02	1.09
Paeoniae Radix	0.78	0.91	0.60	1.52	0.88	0.88	0.86	1.03
Arctii Fructus	0.88	0.93	0.67	1.39	0.68	0.74	0.66	1.13
Polygalae Radix	0.75	0.73	0.57	1.29	0.87	0.85	0.85	1.01
Glehniae Radix cum Rhizoma	0.85	0.75	0.65	1.16	0.93	0.93	0.91	1.02
Sinomeni Caulis et Rhizoma	0.77	0.78	0.58	1.33	0.91	0.89	0.88	1.01
Dipsaci Radix	0.99	0.99	0.76	1.31	0.94	0.91	0.91	1.00
Achyranthis Radix	0.84	0.60	0.64	0.93	0.96	0.95	0.93	1.02
Leonuri Herba	0.89	0.77	0.68	1.14	0.94	1.18	0.91	1.29
Woodfordidia fruticosa[a]	0.68	0.62	0.52	1.19	0.50	0.49	0.48	1.02
Quercus infectoria	0.48	0.47	0.37	1.26	0.41	0.34	0.40	0.86
Gardenia oronaria	0.77	0.60	0.59	1.02	0.82	0.76	0.80	0.96
Penthum Operculina	0.83	0.69	0.63	1.09	0.88	0.87	0.85	1.01
Iris florentia	0.85	0.69	0.65	1.06	0.78	0.76	0.76	1.00
Myrica nagi	0.82	0.76	0.63	1.21	0.82	0.83	0.79	1.05
Chamaecyparis obtusa (MeOH Ext., upper layer)	0.18	0.17	0.14	1.24	0.24	0.27	0.24	1.13

Table I. Continued.

Extract	MDA-MB-231		Predicted additive effect	Synergy Index	MDA-MB-468		Predicted additive effect	Synergy Index
	TRAIL (-)	TRAIL (+)			TRAIL (-)	TRAIL (+)		
	1.00	0.76			1.00	0.97		
Chamaecyparis obtusa (MeOH Ext., lower layer)	0.72	0.21	0.55	0.39	0.75	0.37	0.73	0.51
Cordyceps sinensis	0.91	0.77	0.70	1.11	1.05	0.88	1.02	0.86
Derris floribumda ($CHCl_3$ Ext.)	0.82	0.55	0.63	0.88	0.87	0.65	0.85	0.77
Derris floribumda (MeOH Ext.)	0.57	0.53	0.44	1.22	0.79	0.65	0.77	0.84
Cinnamomum obtusifolium Nees.	0.89	0.39	0.68	0.58	0.97	0.68	0.94	0.72
Cryptomeria japonica	0.94	0.26	0.71	0.37	0.66	0.39	0.64	0.61
Uvaria dac	1.13	0.47	0.86	0.54	1.09	0.74	1.06	0.70
Artemisia vulgaris	0.87	0.34	0.66	0.52	0.93	0.35	0.90	0.39
Scoparia dulsis	0.54	0.12	0.41	0.30	0.57	0.36	0.56	0.65
Unidentified	1.01	0.88	0.77	1.14	0.82	0.83	0.80	1.04
Polygonum barbatum (L.)	0.92	0.88	0.70	1.26	0.81	0.78	0.79	0.99
Euphorbia hirta L.	0.75	0.63	0.57	1.11	0.84	0.75	0.81	0.93
Eclipta prostrata	0.62	0.26	0.47	0.55	0.66	0.59	0.64	0.93
Chinopodium ambrosioides	1.05	0.82	0.80	1.03	0.81	0.87	0.79	1.10
Senna alata	1.05	0.86	0.80	1.07	0.84	0.78	0.82	0.96
Allium wallichii	0.62	0.55	0.47	1.18	0.60	0.62	0.59	1.05
Potentilla peduncularis	1.10	0.84	0.84	1.00	0.67	0.77	0.65	1.18
Cortia depressa	0.97	0.44	0.74	0.60	0.81	0.57	0.79	0.72
Rhododendron campanulatum	0.85	0.97	0.65	1.49	0.78	0.77	0.76	1.02
Rhododendron ciliatum Hook.	0.90	0.75	0.69	1.09	0.81	0.79	0.79	1.00
Primula rotundifolia	0.93	0.77	0.71	1.09	1.17	1.06	1.13	0.93
Boschniakia himalaica	1.05	0.94	0.80	1.18	1.09	1.05	1.06	0.99
Clematis montana	1.04	1.04	0.79	1.31	1.18	1.11	1.15	0.97
Bistorta macrophylla	1.07	1.05	0.82	1.29	1.10	1.09	1.07	1.02
Primula denticulata	0.90	0.90	0.69	1.30	1.08	1.02	1.05	0.98
Ohiglossum valgatum	0.77	0.28	0.59	0.48	0.57	0.26	0.55	0.47
Unidentified	0.99	0.97	0.76	1.28	1.13	1.13	1.10	1.03
Semecarpus anacardium	0.15	0.15	0.11	1.30	0.14	0.14	0.14	1.02
Dichasia bengalensis	0.82	0.42	0.63	0.67	0.81	0.37	0.79	0.47
Senna fistula	0.97	0.93	0.74	1.25	1.25	1.10	1.21	0.91
Euphorbia hirta	0.97	0.86	0.74	1.16	1.17	1.04	1.14	0.91
Curcuma caesia	0.08	0.08	0.06	1.31	0.08	0.07	0.07	1.02
Cedrus deodara	0.74	0.63	0.56	1.12	0.96	0.83	0.93	0.89
Argemonum	1.10	0.72	0.84	0.86	0.94	0.79	0.91	0.87
Chrysenthamum	0.73	0.57	0.56	1.02	0.84	0.78	0.81	0.96
Adiantum venustum	0.92	0.53	0.70	0.75	0.77	0.51	0.75	0.69
Nerium oleander	0.97	0.92	0.74	1.25	1.00	0.92	0.97	0.94
Euphorbia royleana	0.08	0.08	0.06	1.27	0.09	0.09	0.09	1.03
Pogostemon benghalensis	0.88	0.36	0.67	0.53	0.65	0.29	0.63	0.45
Bauhinia variegata	0.88	0.89	0.67	1.32	0.91	0.77	0.88	0.87
Tamarindus indica	0.59	0.58	0.45	1.28	0.64	0.57	0.62	0.92
Woodforidia fruticosa[a]	0.59	0.58	0.45	1.28	0.70	0.63	0.68	0.93
Argemone mexicana	0.72	0.56	0.55	1.03	0.51	0.39	0.50	0.80
Ficus lacor	0.72	0.79	0.55	1.43	0.67	0.67	0.65	1.02
Oroxylum indicum	0.78	0.36	0.60	0.61	0.58	0.26	0.56	0.46
Lathyrus sativus	0.61	0.15	0.47	0.33	0.44	0.16	0.43	0.37
Dichroa febrifuga	0.85	0.77	0.65	1.19	0.93	0.89	0.90	0.99

Table I. Continued.

Extract	MDA-MB-231		Predicted additive effect	Synergy Index	MDA-MB-468		Predicted additive effect	Synergy Index
	TRAIL (-)	TRAIL (+)			TRAIL (-)	TRAIL (+)		
	1.00	0.76			1.00	0.97		
Origanum majorana	0.92	0.93	0.70	1.33	1.04	0.98	1.01	0.98
Calotropis gigantea	0.62	0.36	0.48	0.75	0.48	0.16	0.46	0.35
Rhus trichocarpa bark	1.03	0.96	0.78	1.23	0.97	0.92	0.94	0.98
Rhus trichocarpa wood	1.00	0.97	0.76	1.27	0.88	0.83	0.85	0.98
Syzygium cumini	1.04	1.15	0.79	1.46	0.73	0.69	0.70	0.98
Artium lappa	0.91	0.83	0.70	1.20	1.14	1.16	1.10	1.05
Rheum austale	0.65	0.65	0.50	1.31	0.68	0.69	0.66	1.05
Nelumbium speciosum	0.82	0.83	0.62	1.34	0.83	0.85	0.80	1.06
Carum copticum	0.55	0.16	0.42	0.39	0.66	0.35	0.64	0.54
Solanum xanthocarpum schrad	0.25	0.30	0.19	1.56	0.10	0.11	0.10	1.09
Vernonia anthelmintica willd	0.11	0.11	0.08	1.29	0.11	0.11	0.11	1.05
Aconitum spicatum	0.94	0.71	0.72	0.99	0.98	0.87	0.95	0.92
Nardostachys grandiflora	0.07	0.07	0.05	1.31	0.08	0.08	0.08	1.01
Delphinium denudatum	0.96	0.81	0.73	1.10	1.11	0.96	1.07	0.89
Kaunia longipetiolata	0.85	0.90	0.65	1.39	0.85	0.82	0.83	0.99
Mucuna nigricans	0.96	0.88	0.73	1.20	1.14	1.07	1.11	0.96
Strychnos nuxvomica	0.92	0.82	0.70	1.16	1.00	0.97	0.97	0.99
Adhatoda vasica Nees	0.96	0.86	0.74	1.16	1.03	0.92	1.00	0.92
Wrightia tinctoria	0.76	0.70	0.58	1.21	0.76	0.54	0.74	0.73
Symplocos racemosa	0.88	0.96	0.67	1.44	0.91	0.87	0.88	0.99
Piper chabo	0.68	0.17	0.52	0.32	0.38	0.15	0.37	0.42
Paris poyphilla	0.25	0.24	0.19	1.21	0.10	0.09	0.09	1.00
Withania somnifera	1.01	1.04	0.77	1.35	1.01	0.89	0.98	0.91
Linum usitatissimum	0.96	0.87	0.73	1.20	0.98	1.00	0.95	1.05
Rhododendron anthopogon	0.82	0.68	0.63	1.08	0.70	0.62	0.68	0.91
Inula cappade	0.96	0.94	0.73	1.29	0.89	0.92	0.86	1.06
Citrullus colocynthis	0.99	0.83	0.75	1.11	0.85	0.88	0.82	1.08
Calotropis gigantean	0.88	0.94	0.67	1.39	0.95	0.87	0.92	0.95
Lyonia ovalifolia	0.99	1.10	0.75	1.47	1.13	1.22	1.10	1.11
Periploca calophylla	0.93	1.04	0.71	1.46	1.31	1.41	1.27	1.11
Zanthoxylum armatum	0.92	1.02	0.70	1.44	1.56	1.40	1.52	0.92
Unidentified	1.02	1.10	0.78	1.42	1.59	1.51	1.55	0.97
Achyranthus aspera	0.96	1.03	0.73	1.40	1.54	1.47	1.49	0.99
Woodforidia fruticosa[a]	0.68	0.67	0.52	1.31	0.86	0.89	0.84	1.06
Ficus lacor	0.91	0.97	0.70	1.40	1.35	1.36	1.31	1.04
Paris polyphylla	0.63	0.70	0.48	1.45	0.42	0.47	0.41	1.15
Solanum xanthocarpum	0.28	0.32	0.22	1.50	0.37	0.32	0.36	0.91
Equisetum debile	1.06	1.07	0.81	1.32	1.00	0.86	0.98	0.89
Pogostemon benghalensis	0.91	1.03	0.69	1.48	1.13	1.15	1.10	1.05
Girardiana heterophylla	0.94	0.85	0.72	1.19	1.04	0.99	1.01	0.97
Gaultheria fragrantisia	0.98	0.92	0.75	1.23	1.07	1.07	1.04	1.03
Crateva unilocularis	1.00	1.04	0.76	1.37	1.23	1.24	1.20	1.04
Rabdosia rugosa	0.99	1.00	0.75	1.33	1.13	1.21	1.09	1.11
Curcuma amada	0.97	0.87	0.74	1.18	0.78	0.62	0.76	0.82
Rhododendron anthopogon	0.82	0.87	0.62	1.40	0.90	0.87	0.87	1.00

[a]These plants were collected under different circumstances as regards the time and place.

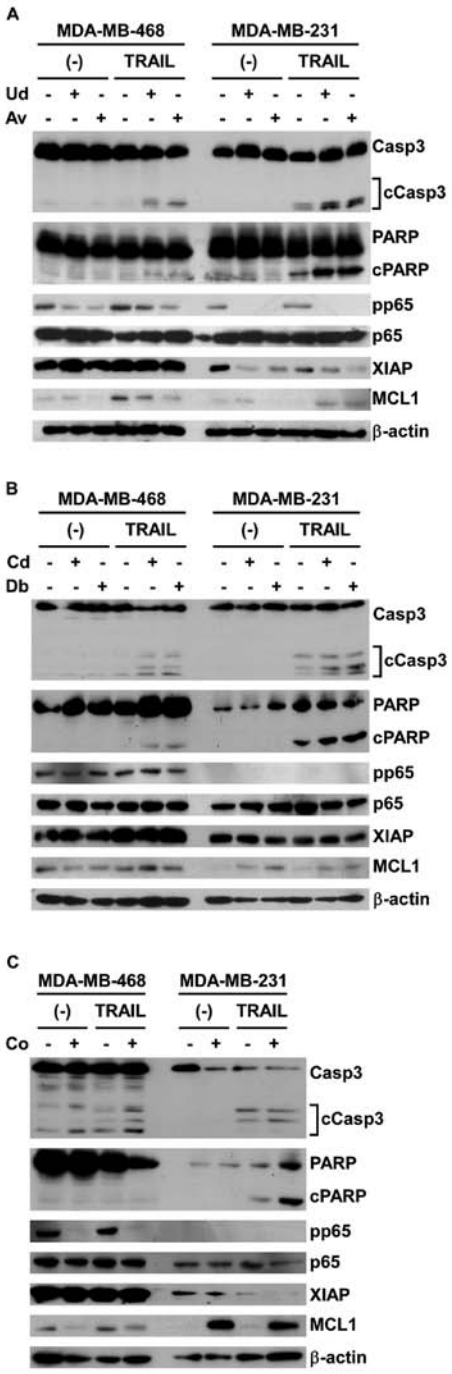


Figure 4. Synergistic effect of the identified medicinal plant extracts on the TRAIL-induced apoptosis. (A-C) Expression of various proteins was detected by western blot analysis in breast cancer cells treated with plant extracts (50 μg/ml) and TRAIL (50 ng/ml). Ud, *U. dac*; Av, *A. vulgaris*; CD, *C. depressa*; Db, *D. bengalensis*; Co, *C. obtusifolium*.

Discussion

In the present study, we screened 138 medicinal plant extracts for their ability to potentiate TRAIL effects in TRAIL-sensitive cells and to overcome resistant mechanisms in TRAIL-insensitive cells. From the screening, 5 potential medicinal plant extracts were identified to sensitize TNBC cancer cells to TRAIL. For identification, we used only two distinct criteria: whether the plant extract displays only minimal toxicity (<20% at 50 μg/ml concentration) and whether the extract potentiates TRAIL-induced cell death in a synergistic manner. Our results demonstrated that these two criteria suffice to identify extracts as TRAIL-sensitizing reagents. This screening criteria can also be applied for drugs other than TRAIL to identify drug-sensitizing reagents.

Although we only focused on plant extracts showing low cytotoxicity and synergistic effects with TRAIL in both cells, the screening results in Fig. 2 and Table I provide significant insight into the cytotoxic activity of each plant extract as well as their combination effects with TRAIL. Eight plant extracts *(C. obtusa, S. dulsis, O. valgatum, O. indicum, L. sativus, C. gigantean, C. copticum* and *P. chabo)* showed cytotoxicity and synergistic effects in both cell lines (Fig. 2). In addition, 38 plant extracts *(*including *S. anacardium, C. caesia, E. royleana*) revealed strong cytotoxicity with or without TRAIL in both cell lines (Table I); therefore, the active constituents present in these extracts need further investigation as potential chemotherapeutic agents against breast cancer. The extracts of *C. fructus, C. japonica, A. venustum* and *P. benghalensis* showed low cytotoxicity with synergistic effects with TRAIL in MDA-MB-231 cells but showed cytotoxicity and synergistic effects in MDA-MB-468 cells. Therefore, further study of these extracts may provide critical information and clues to identify TRAIL-resistant mechanisms that are specifically active in MDA-MB-468 cells.

Among the 5 plant extracts sensitizing breast cancer cells to TRAIL, *U. dac* and its active constituent (+) grandifloracin showed anti-austerity activity against PANC-1 human pancreatic cancer cells in a nutrition-deficient tumor-mimicking environment (18). The extract of *A. vulgaris* has been reported to have cytotoxicity against MCF-7 cells (19). Consistent with these findings, *U. dac* and *A. vulgaris* also showed cytotoxicity at higher concentrations (Fig. 3A and B) in the present study, although we used lower non-cytotoxic concentrations to identify the synergistic effects with TRAIL. The possible mechanisms of sensitization by the newly identified plant extracts to TRAIL-induced cell death of TNBC were investigated by western blot analysis (Fig. 4). In particular, *U. dac*, *A. vulgaris* and *C. obtusifolium* may sensitize breast cancer cells via suppression of the phosphorylation of p65, which is involved in the resistant mechanism to TRAIL (9); however, further investigation of the other two extracts, *C. depressa* and *D. bengalensis*, is necessary to understand the mechanism of action of these extracts.

In conclusion, the results of this study provide valuable information about potential medicinal plants that could enhance TRAIL activity and develop combination therapies against breast cancer. The 5 medicinal plant extracts identified in the present study were unique, as they enhanced the sensitivity to TRAIL in both TRAIL-sensitive and -insensitive TNBC cells. Furthermore, these extracts could have potential medical applications to improve TRAIL efficacy and to overcome resistance mechanisms not only in breast cancer, but in other types of cancer as well.

Acknowledgements

We thank Professor Hiroaki Sakurai (Faculty of Pharmaceutical Sciences, University of Toyama, Japan) for helpful discussions.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global Cancer Statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Brenton JD, Carey LA, Ahmed AA and Caldas C: Molecular classification and molecular forecasting of breast cancer: ready for clinical application? J Clin Oncol 23: 7350-7360, 2005.
3. Hudis CA and Gianni L: Triple-Negative Breast Cancer: An Unmet Medical Need. Oncologist 16: 1-11, 2011.
4. Haffty BG, Yang Q, Reiss M, *et al*: Locoregional relapse and distant metastasis in conservatively managed triple negative early-stage breast cancer. J Clin Oncol 24: 5652-5657, 2006.
5. Dent R, Trudeau M, Pritchard KI, *et al*: Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13: 4429-4434, 2007.
6. Keane MM, Ettenberg SA, Nau MM, Russell EK and Lipkowitz S: Chemotherapy augments TRAIL induced apoptosis in breast cell lines. Cancer Res 59: 734-741, 1999.
7. Chinnaiyan AM, Prasad U, Shankar S, *et al*: Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc Natl Acad Sci USA 97: 1754-1759, 2000.
8. Walczak H, Miller RE, Ariail K, *et al*: Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat Med 5: 157-163, 1999.
9. Lirdprapamongkol K, Sakurai H, Suzuki S, *et al*: Vanillin enhances TRAIL-induced apoptosis in cancer cells through inhibition of NF-κB activation. In Vivo 24: 501-506, 2010.
10. Lirdprapamongkol K, Sakurai H, Kawasaki N, *et al*: Vanillin suppresses in vitro invasion and in vivo metastasis of mouse breast cancer cells. Eur J Pharm Sci 25: 57-65, 2005.
11. Choo MK, Kawasaki N, Singhirunnusorn P, *et al*: Blockade of transforming growth factor-beta-activated kinase 1 activity enhances TRAIL-induced apoptosis through activation of a caspase cascade. Mol Cancer Ther 5: 2970-2976, 2006.
12. Falschlehner C, Emmerich CH, Gerlach B and Walczak H: TRAIL signalling: decisions between life and death. Int J Biochem Cell Biol 39: 1462-1475, 2007.
13. Burns TF and El-Deiry WS: Identification of inhibitors of TRAIL-induced death (ITIDs) in the TRAIL-sensitive colon carcinoma cell line SW480 using a genetic approach. J Biol Chem 276: 37879-37886, 2001.
14. Clohessy JG, Zhuang J, de Boer J, Gil-Gómez G and Brady HJ: Mcl-1 interacts with truncated Bid and inhibits its induction of cytochrome c release and its role in receptor-mediated apoptosis. J Biol Chem 281: 5750-5759, 2006.
15. Chawla-Sarkar M, Bae SI, Reu FJ, Jacobs BS, Lindner DJ and Borden EC: Downregulation of Bcl-2, FLIP or IAPs (XIAP and survivin) by siRNAs sensitizes resistant melanoma cells to Apo2L/TRAIL-induced apoptosis. Cell Death Differ 11: 915-923, 2004.
16. Ravi R and Bedi A: Potential methods to circumvent blocks in apoptosis in lymphomas. Curr Opin Oncol 14: 490-503, 2002.
17. Shrader M, Pino MS, Lashinger L, Bar-Eli M, Adam L, Dinney CP and McConkey DJ: Gefitinib reverses TRAIL resistance in human bladder cancer cell lines via inhibition of AKT-mediated X-linked inhibitor of apoptosis protein expression. Cancer Res 67: 1430-1435, 2007.
18. Awale S, Ueda J, Athikomkulchai S, Abdelhamed S, Yokoyama S, Saiki I and Miyatake R: Antiausterity agents from *Uvaria dac* and their preferential cytotoxic activity against human pancreatic cancer cell lines in a nutrient-deprived condition. J Nat Prod 75: 1177-1183, 2012.
19. Taghizadeh Rabe SZ, Mahmoudi M, Ahi A and Emami SA: Antiproliferative effects of extracts from Iranian *Artemisia* species on cancer cell lines. Pharm Biol 49: 962-969, 2011.