Artemisia princeps var *orientalis* Induces Apoptosis in Human Breast Cancer MCF-7 Cells

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Abstract. *Background: Dried leaves of Artemisia princeps var orientalis are used in the Eastern practice of moxibustion to improve general health. The ability of A. princeps smoke and water extracts to induce apoptosis was evaluated in human breast cancer MCF-7 cells in vitro. Materials and Methods: Tumor cells were cultured with a smoke or water extract (1.5-50% v/v) for 72 h, and cytotoxicity and apoptosis were determined by MTT and TUNEL assays, respectively. Activation of caspases, changes in membrane potential, and BCL-2 expression were determined by flow cytometry. Results: Both preparations inhibited the growth of breast cancer cells in a dose-dependent manner. Induction of apoptosis was associated with activation of caspases 3, 8 and 9, depolarization of the mitochondrial membrane potential and down-regulation of BCL-2 expression. Furthermore, A. princeps smoke exerted synergistic cytotoxicity with doxorubicin. Conclusion: The data suggest that A. princeps smoke and water soluble extracts induce apoptosis via the mitochondrial pathway and may represent a novel adjuvant for the treatment of breast cancer.*

Among all types of cancer, breast cancer is the second leading cause of death in women. A prerequisite for successful treatment of breast cancer is the susceptibility of the cancer cells to apoptosis. Indeed, most of the current conventional approaches to breast cancer treatment, including radiation, chemotherapy, and biological hormonal therapy with tamoxifen and Herceptin™, induce apoptosis in breast cancer cells (1-6). Despite significant advances in primary and adjuvant treatment for local breast cancer, many patients suffer a systemic relapse. Therefore, there is a need to identify new agents that induce apoptosis and to develop complementary and/or alternative treatments for the treatment of breast cancer.

Apoptosis is an orchestrated form of cell death. Abnormal apoptosis is associated with a wide variety of human diseases such as cancer, auto-immune disease and neurodegenerative diseases. The two most common apoptotic pathways are the extrinsic and the intrinsic pathway (mitochondrial pathway) (7, 8). The extrinsic pathway is initiated by stimulation of death receptors (*e.g.* CD95, TNFR-1) in the plasma membrane (9, 10). The intrinsic pathway is initiated by chemotherapeutic agents, radiation and other cellular stresses that disrupt mitochondrial integrity (11, 12). Both apoptotic pathways culminate in the activation of caspases, a family of intracellular cysteine proteases which can disrupt cells within a few minutes from their activation (13, 14).

Artemisia princeps var *orientalis*, belonging to the asteracea family, is widely used in Eastern medicine for the treatment of circulatory disorders, such as dysmenorrhea, haematuria, hemorrhoids, inflammation, and also for the treatment of chronic conditions, such as cancers, ulcers and digestive disorders. Dried leaves of this herb are also used in the Eastern practice of moxibustion to improve general health. Water extracts of *A. princeps* have been shown to modulate mouse thymocyte apoptosis by regulating the expression of death receptor (Fas/CD95) (15). The aim of this study was to investigate the potential apoptotic activity of this plant's extracts against human breast carcinoma cells.

Materials and Methods

Cells. The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. These tumor cells were maintained in RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 100 μg/ml streptomycin and penicillin. Peripheral blood lymphocytes (PBL) were separated from peripheral blood obtained from healthy young volunteers by Ficoll-Hypaque (Mediatech, Inc., Herndon, VA, USA) density gradient centrifugation. Lymphocytes were resuspended in RPMI-1640 medium (GIBCO, Grand Island,

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Key Words: Artemisia princeps, breast cancer cells, anticancer activity, apoptosis.

NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Tested substances. Dried leaves of *Artemisia princeps* var *orientalis*, rice straw and pine cones were supplied by Gangwha Mugwort Farming, Gangwondo, Dongum Ri, South Korea. Two types of extracts were tested: i) smoke from the burnt leaves, and ii) water extracts from the ash of burnt *Artemisia* leaves.

Preparation of Artemisia smoke. Six grams of dried leaves of *A. princeps* or rice straw were burned in a moxibustion apparatus (Moxa Institute, Los Angeles, CA, USA) and the smoke was collected in 50 ml complete culture medium. Rice straw smoke was used as a control.

Preparation of A. princeps extract in water. Twelve grams of ash derived from burning the dried leaves of *A. orientalis* or pine cone were mixed with 1.5 liters of water and boiled for 2.5 hours. Undissolved ash was allowed to settle for 24 hours, and the sediment was removed by filtration. Ash water was buffered by adding 1 ml of 10xRPMI medium to 9 mL of *A. princeps* extract and was used in experiments. As a control, water extracts of pine cones were used.

Assessment of apoptosis. Cell death and apoptosis were assessed using [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assays, respectively. The MTT assay is based on the reduction of tetrazolium salt, MTT, by a mitochondrial dehydrogenase of viable cells to a blue-colored formazan product that can be measured by spectrometry (16, 17). The amount of formazan produced is proportional to the number of living cells.

MCF-7 cells and PBL were seeded in 96-well flat bottom plates at $2x10^4$ cells per well and were cultured in triplicate in the presence or absence of different concentrations (1.25% -25%) of water soluble extract or smoke of *A. princeps*. MCF-cells were also incubated with or without different concentrations (0.001 μM to 5 μM) of doxorubicin (ADR, Sigma Chemical Company, St. Louis, MO, USA) in the presence or absence of 2.5% of *A. princeps* smoke. The cultures were incubated at 37˚C for 72 hours, after which 50 μg of MTT was added to each well, and the cultures were additionally incubated for 4 hours. The plates were centrifuged, the medium carefully removed, the formazan crystals solubilized with acid alcohol, and the plates read at 590 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA, USA). The 50% inhibitory concentration (IC_{50}) was defined as the concentration of drug that caused a 50% reduction in absorption.

TUNEL assay. DNA strand breakage was measured using the TUNEL assay. Briefly, MCF-7 cells ($2x10^5$ cells/ml) were incubated at 37˚C with or without *A. princeps* extracts. After 48 hours of incubation, cells were fixed with 2% formaldehyde, washed with phosphate-buffered saline (PBS) and permeabilized with 2% sodium citrate and 10% Triton X-100 for 5 minutes on ice. After washing, cells were incubated with FITC-dUTP (Boehringer Mannheim, Indianapolis, IN, USA) in the presence of terminal dNTP transferase enzyme solution containing 1 μM potassium cacodylate and 125 μM tris-HCL (pH-6.6; *In Situ* Detection Kit; Boehringer Mannheim) for 1 hour at 37˚C. After incubation, cells were washed with PBS, and 10,000 cells were acquired and analyzed using FACScan with Cell Quest software (Becton Dickinson, Menlo Park, CA, USA).

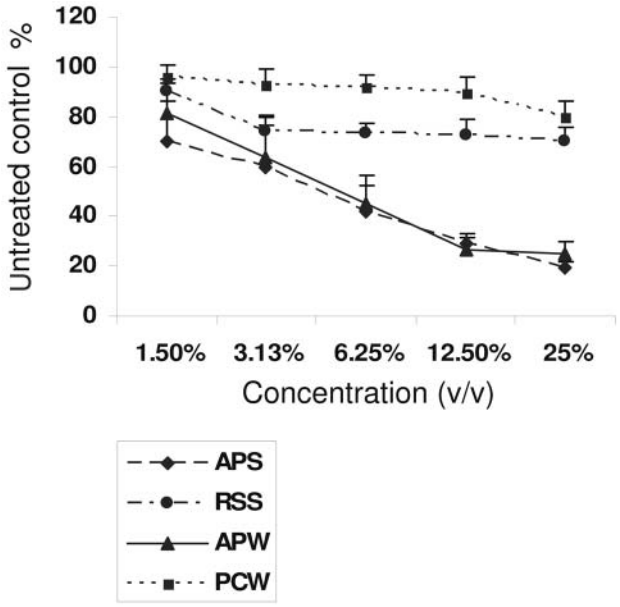

Figure 1. *Effect of smoke and water extracts of A. princeps on the survival of breast cancer cells. MCF-7 cells were cultured with smoke or water extracts for 3 days and cytotoxicity was determined by MTT assay. APS: A. princeps smoke, RSS: rice straw smoke, APW: A. princeps water extract, PCW: pine cone water extract.*

Mitochondrial potential $\Delta\psi_m$. Changes of the mitochondrial transmembrane potential $\Delta\psi_m$ during apoptosis were studied using 3'3'-dihexyloxacarbocyanine dye [$DIOC_6$ (3)] (Molecular Probes, Eugene, OR, USA) as described elsewhere (18). This cyanine dye accumulates in the mitochondrial matrix under the influence of the $\Delta\psi_m$. MCF-7 cells that were treated for 24 hours with *A. princeps* smoke were incubated with 0.5 mm $DIOC_6$ (3) for 30 minutes at 37˚C. Cells were transferred on ice for FACS analysis. Forward and side scatters were used to gate and exclude cellular debris using a FACScan. Cells were excited at 488 nm and green fluorescence was collected on FL1 at 530 nm. Ten thousand cells were analyzed. Data was acquired and analyzed using Cell Quest software (Becton Dickinson).

Expression of CD95 and BCL-2. MCF-7 were exposed to *A. princeps* smoke for 48 hours. Treated and untreated cells were stained with PE-labeled anti-human CD95 antibody or isotype control IgG (B.D. Biosciences) and the surface expression of CD95 was determined by flow cytometry. For detection of BCL-2, cells were first fixed and permeabilized with ice-cold 70% methanol. They were then stained with FITC labeled anti-BCL-2, or isotype control (Dako Corp, Carpentaria, CA, USA). Cells were washed and analyzed using FACScan. The percentages of cells expressing CD95, BCL-2 and mean fluorescence intensity (an indicator of density of molecules/cell) were determined.

Table I. *Effect of smoke and water extracts of A. princeps on the survival of peripheral blood lymphocytes.*

Concentration (v/v)	Smoke O.D	Water extract O.D
0	0.24±0.01	
1.5	0.29±0.04	0.28±0.03
3.125	0.27±0.01	0.20±0.01
6.25	0.26±0. 01	0.25±0.01
12.5	0.26±0. 01	0.23±0.01
25	0.28±0.02	0.27±0.01

Peripheral blood mononuclear cells were cultured with the indicated concentrations of smoke and water extracts of *A. princeps* for 3 days. Lymphocyte survival was determined by the MTT assay. The O.D values represent mean±SD of triplicate cultures.

Intracellular activity of caspases 3, 8 and 9. The method is based on carboxyfluorescein-labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-toxic. Once inside the cells, these inhibitors bind covalently to the active caspase. Caspase-positive (þ) cells are distinguished from caspase-negative (2) cells with the aid of flow cytometry. Briefly, cells undergoing apoptosis were loaded with labeled FMK-peptide inhibitors (FAM-LETD-FMK for caspase-8, FAM-LEHD-FMK for caspase-9, and FAM-DEVD-FMK for caspase-3; Cell Technology Inc., Mountain View, CA, USA). After 1 hour of incubation, the cells were washed to remove unbound caspase, and cells that contained bound inhibitors were quantified using a FACScan flow cytometer.

Statistical analysis. Statistical analysis of histograms was performed using the Kolmogorov-Smirnov statistics. A D value of 0.2 was considered statistically significant.

Results

Cytotoxicity of Artemisia princeps smoke and water soluble extracts. MCF-7 cells were cultured with different amounts of *A. princeps* smoke and water soluble extracts and cytotoxicity was determined *via* MTT assay. Results are shown in Figure 1. The data show that smoke and water extracts of *A. princeps* inhibited the growth of breast cancer cells in a dose dependent manner. The IC_{50} values for smoke (4±2%) and water extracts (5±2.5%) were essentially similar. The data in Figure 1 also show that smoke derived from rice straw and water extracts of pine cone had no effect on the survival of MCF-7 cells, suggesting the observed effects were specific for *A. princeps*. To examine the cell specificity, peripheral blood lymphocytes (PBL) were cultured with *A. princeps* smoke and water soluble extracts, and their survival was determined. It can be seen from the results in Table I that smoke and water extracts were not toxic to normal blood lymphocytes, suggesting that the cytotoxic-inducing activity of smoke and water soluble extracts were selective for cancer cells.

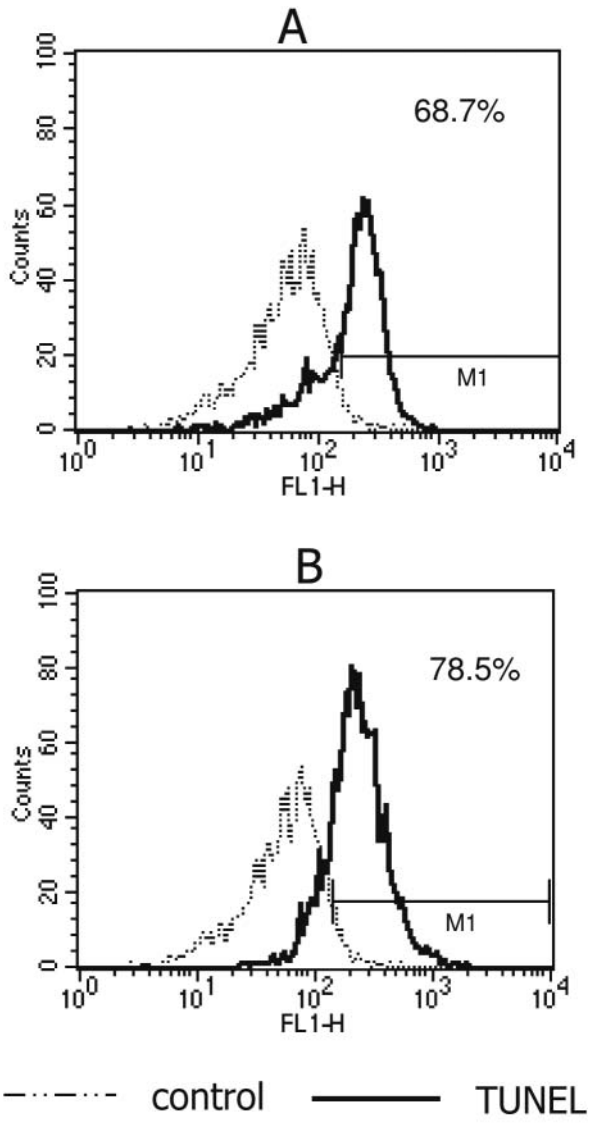

Figure 2. *Effect of A. princeps smoke on MCF-7 cell apoptosis. Cells (MCF-7) were incubated with 5% (A) or 10% (B) smoke of A. princeps for 24 hours. Apoptotic cells were determined by TUNEL technique using FACScan flow cytometer. A representative histogram showing increased apoptosis in cells cultured with smoke.*

A. princeps smoke induces apoptosis in breast cancer cells. One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. To determine whether the observed cytotoxicity was due either to apoptosis or necrosis, MCF-7 cells treated with smoke were analyzed by *in situ* nick-end labeling (TUNEL) staining that detects cells containing 3' end fragmented DNA produced as a result of apoptotic cell death. It can be seen from the FACS histogram data presented in Figure 2 that an increased proportion of MCF-7 cells underwent apoptosis as indicated by increased TUNEL-positive cells after exposure to *A. princeps* smoke.

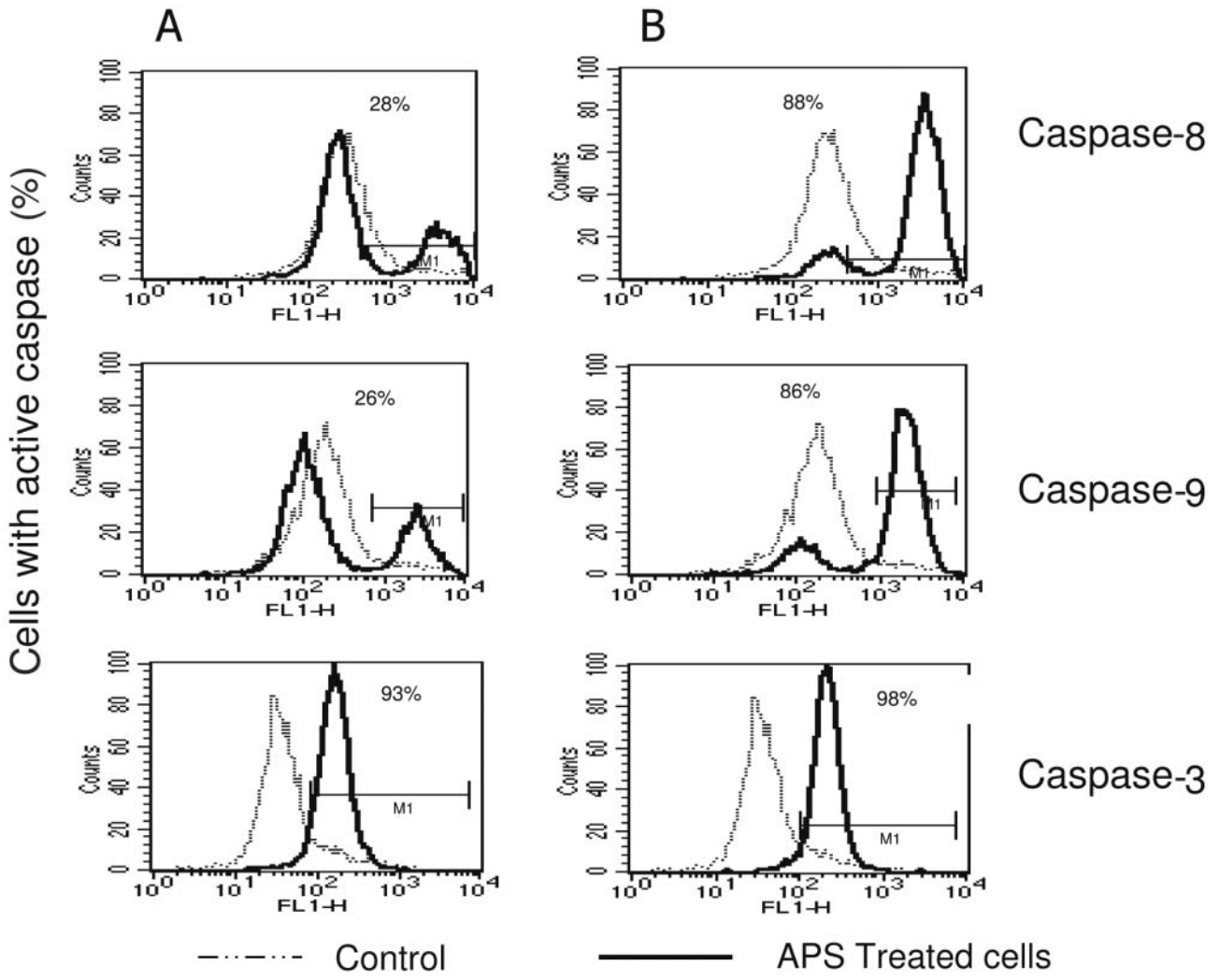

Figure 3. *Increased activation of caspases 3, 8 and 9. Cells were treated with 5% (A) or 10% (B) A. princeps, smoke and intracellular active caspases 3, 8 and 9 were determined with a caspase-3, -8 and -9 determination kit using FACScan. APS: A. princeps smoke.*

A. princeps smoke activates caspases 3, 8 and 9. Apoptosis is mediated by activation of the caspase cascade. In order to determine the steps in *A. princeps* smoke-mediated apoptosis, we examined the activation of proximal caspases (caspase-8, caspase-9) and the executioner caspase (caspase-3). The data in Figure 3 is a representative histogram plot for activation of caspases. The data show that *A. princeps* induced the activation of caspase-3, -8, and -9 in MCF-7 cells.

A. princeps smoke does not alter the level of expression of the death receptor CD95. To determine whether the observed apoptosis of breast cancer cells is due to upregulation of a death receptor, CD95 expression on MCF-7 cells treated with and without *A. princeps* smoke was determined. It was observed that the percentage of cells expressing CD95 and the density of CD95 on MCF-7 treated cells was similar to that of untreated cells (Figure 4). These results suggest that the *A. princeps* extract induces apoptosis *via* a death receptor independent, intrinsic pathway.

A. princeps smoke disrupts mitochondrial membrane potentials. Activation of the intrinsic pathway of apoptosis leads to the disruption of the mitochondrial membrane potential and to the release of mitochondrial contents. This results in activation of caspase-9 and -3. (8). To characterize the apoptosis-inducing activity of *A. princeps* smoke, we determined mitochondrial membrane potentials. The data in Figure 5 show that MCF-7 cells treated with *A. princeps* extract exhibited lower membrane potentials than untreated cells.

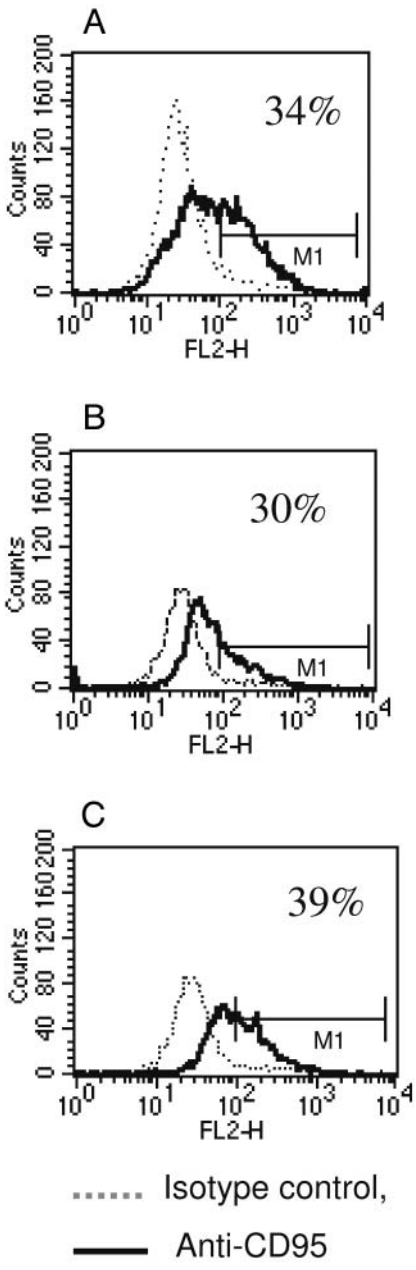

Figure 4. *Effect of A. princeps smoke on CD95 expression. MCF-7 cells were incubated without (A) or with 5% (B) or 10% (C) A. princeps smoke for 24 hours. The cells were then stained with PE conjugated anti-CD95 or isotype control and were subjected to flow cytometry analysis to determine the expression of CD95. The numbers in the histogram represent the proportion of cells expressing CD95. The mean fluorescence channel numbers (a measure for the density of receptors) for untreated and cells treated with 5% and 10% of A. princeps smoke were 125, 110 and 131, respectively. These values were not significantly different (D≤0.2).*

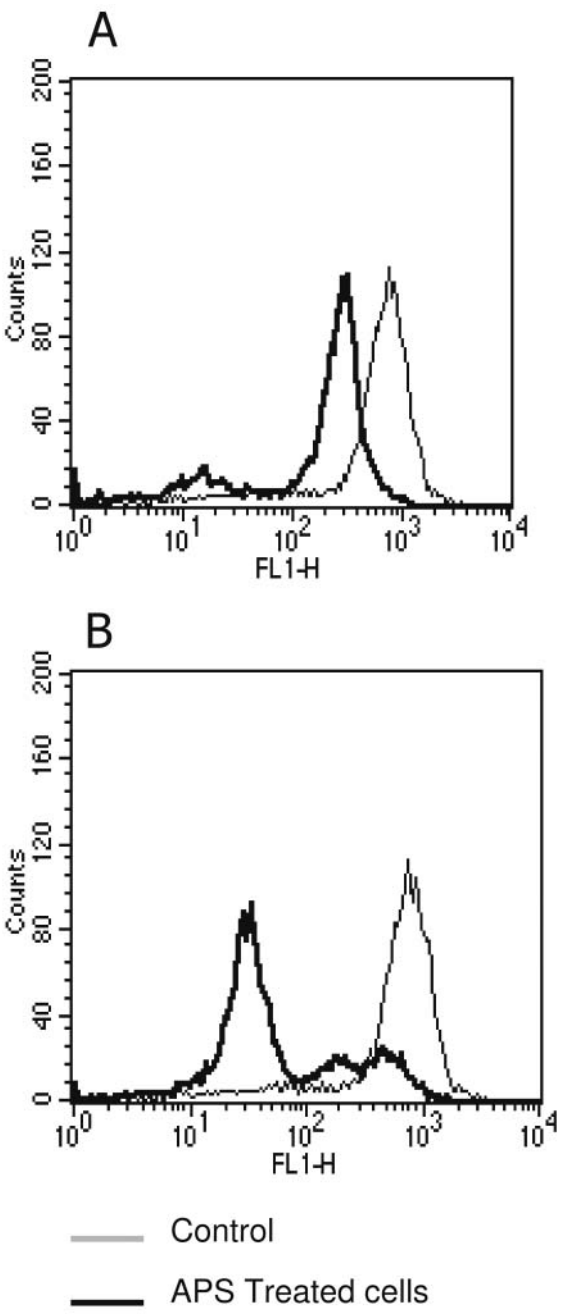

Figure 5. *Effect of A. princeps smoke on mitochondrial potential. MCF-7 cells were incubated with or without A. princeps smoke for 24 hours. The cells were then stained with $DIOC_6$ and were subjected to flow cytometry analysis to determine the $\Delta\psi_m$. The numbers in the histogram represent the proportion of cells exhibiting a decreased membrane potential.*

A. princeps smoke down-regulates BCL-2 expression. BCL-2 is an anti-apoptotic molecule that is shown to protect cells from apoptosis induced by various agents that disrupt the mitochondrial membrane potential. To further investigate the effect of *A. princeps* extracts, we examined its effect on BCL-2 expression (Figure 6). As expected, MCF-7 cells

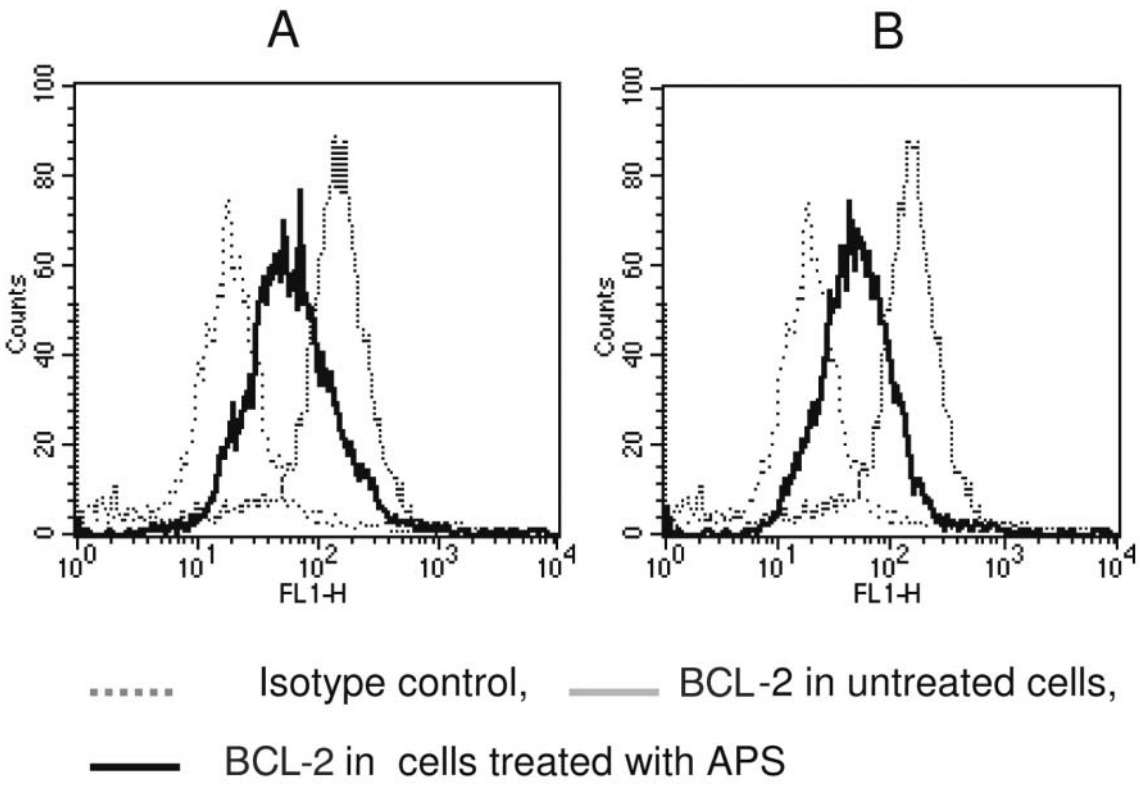

Figure 6. *MCF-7 cells (1x10^5 cells/ml) were cultured in the absence or presence of 5% (A) or 10% (B) A. princeps smoke for 24 hours. Expression of BCL-2 was determined by staining the cells with anti-human BCL-2 antibody and flow cytometry. Isotype control (MFC=mean fluorescence channel number). Significantly different from control untreated cells, D=0.30.*

(97%) expressed BCL-2 and the density (as measured by mean fluorescence intensity, MFC) was 193 channels. Smoke of *A. princeps* reduced significantly (D=0.3) both the percentage of cells expressing BCL-2 and the density of BCL-2 expression: the proportion of BCL-2 expressing MCF-7 cells cultured in the presence of 5% and 10% *A. princeps* smoke was 59% (MFC#129) and 41% (MFC#107), respectively.

A. princeps smoke potentiates cytotoxicity of doxorubicin. The efficacy of a combined treatment of *A. princeps* smoke and doxorubicin, a conventional chemotherapeutic agent against breast cancer cells (Adriamycin, ADR), in killing MCF-7 cells was examined. As expected, doxorubicin alone at 0.75 μM concentration significantly reduced the number of MCF-7 cells in the culture by 50% (Figure 7). *A. princeps* smoke (2.5%) alone at 72 hours reduced the number of MCF-7 cells by approximately 15% compared to the non-treated control cells. The addition of smoke to doxorubicin resulted in a 2.7-fold reduction in the IC_{50} value of doxorubicin (IC_{50} of smoke+ADR=0.27 μM).

Discussion

Susceptibility to apoptosis is a prerequisite for successful treatment of cancer cells with chemotherapy, radiation therapy, natural killer cells or cytotoxic T-cells. The aim of this study was to investigate whether human breast cancer cells undergo apoptosis when exposed to *Artemisia princeps* var *orientalis* extracts. To this aim, we exposed human breast cancer MCF-7 cells to *A. princeps* smoke and water extracts, and apoptosis was determined by assessing cell survival, DNA fragmentation and activation of caspases. In our experimental conditions, we observed that *A. princeps* smoke and water extract induced apoptosis in breast cancer cells in a dose dependent manner, but had no detectable cytotoxic effect on human peripheral blood lymphocytes. This anticancer effect is selective in that smoke and water extracts derived from pine cone and grass failed to induce apoptosis in breast cancer cells. These results suggest that *A. princeps* var *orientalis* smoke and water extracts contain bioactive constituents that exert a pro-apoptotic activity against human breast cancer cells.

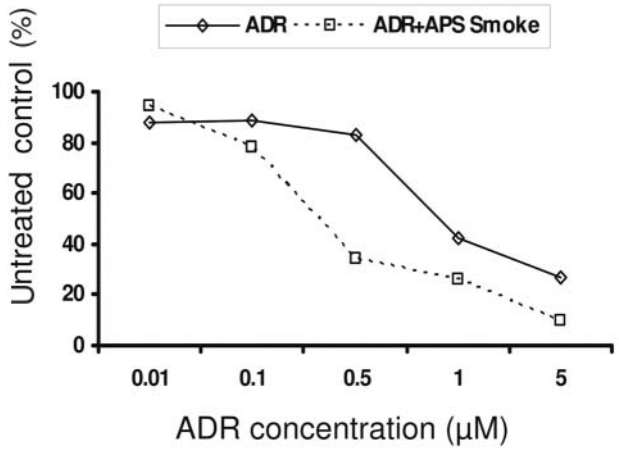

Figure 7. *Effect of A. princeps smoke on adriamycin cytotoxicity in MCF-7 cells. MCF-7 cells were cultured with A. princeps smoke for 3 days in the presence or absence of ADR (0.01 μM to 5 μM). Cell survival was determined using an MTT assay. Cells + DNR (◇), cells + ADR+ smoke (□).*

The genus *Artemisia* consists of more than 350 species, and plant extracts derived from some of the species have been used for centuries in traditional Eastern medicine for the treatment of various ailments including malaria, inflammation, hepatitis and infections caused by microorganisms. Dried leaves of this herb are also used in the Eastern practice of moxibustion. Hitosugi *et al.* (19) reported that *Artemisia capillaris* smoke and aqueous extracts induced apoptosis in the human myelogenous leukemia cell line, HL-60, but not in human breast cancer MCF-7 cells or other tumor cells. Hu *et al.* (20) showed that macromolecular components of *A. capillaris* induce apoptosis in human hepatoma cell lines, and Shoemaker *et al.* (21) reported water soluble extracts of *A. argyi* were less active against human tumor cell lines, including breast cancer cell lines, as compared to murine tumor cells. Flavones derived from *A. argyi* and *A. asiatic* have been reported to inhibit the growth of human lung cancer, prostate cancer, melanoma, myeloid leukemia, and gastric cancer cell lines *via* inducing apoptosis (22-25). However, these compounds were found to be ineffective against human breast cancer cell lines (23). Artemesinin and related compounds derived from *A. annua* have been shown to inhibit the growth of human colorectal and breast cancer cells (26, 27). Our findings, taken together with the above reports, would suggest that some but not all species of *Artemisia* possess biologically active components against breast cancer and that screening of other *Artemisia* species may help identify novel anticancer compounds active against breast cancer.

To investigate the molecular mechanism(s) by which *A. princeps* var *orientalis* smoke mediates apoptosis, the effect of smoke on known effector molecules in death receptor and mitochondrial pathways of apoptosis was examined. In this study, we have shown that smoke had no effect on the expression of death receptor CD95, suggesting that smoke exerts its activity by a mechanism independent of upregulation of the death receptor. Chung *et al.* (15) reported that a carbohydrate extracted from a mixture of dried *Artemisia* species down-regulated the expression of CD95 in mouse thymocytes and prevented their apoptosis. The discrepancy between our results and that of Chung *et al.* (15) may be related to the cell type used, *i.e.* cancer cells *vs.* normal mouse thymocytes. In this study, we found that smoke had no effect on the survival of normal human lymphocytes, which suggests some specificity of *A. princeps* smoke for breast cancer cells.

Apoptosis that proceeds *via* the mitochondrial pathway involves mitochondrial membrane permeabilization (MMP) (8). Mitochondria contain two well-defined compartments: the matrix surrounded by the inner membrane (IM) and the intermembrane space, surrounded by the outer membrane (OM). The IM contains various molecules, including ATP synthase, the electron transport chain, and adenine nucleotide translocator. Under physiological conditions, these molecules allow the respiratory chain to create an electrochemical gradient or membrane potential ($\Delta\psi_m$). IM permeabilization leads to changes in $\Delta\psi_m$ (28, 29). BCL-2 is located on the IM and appears to play an important role in the maintenance of the mitochondrial membrane potential ($\Delta\psi_m$). The intermembrane space contains cytochrome *c*, certain pro-caspases and apoptosis-inducing factor (AIF). The permeabilization of the mitochondrial membranes results in the release of proapoptotic molecules into the cytosol. The release of cytochrome c triggers the assembly of Apaf-1 (apoptotic protease-activating factor) and pro-caspase-9 to form an apoptosome (8, 28). Pro-caspase-9 is dimerized and activated, and active caspase-9 activates executioner caspases to orchestrate apoptosis. In this study, we showed that *A. princeps* var *orientalis* smoke caused a significant decrease in mitochondrial membrane potential and also caused a significant decrease in the level of expression of the anti-apoptotic protein BCL-2, which is shown to protect the cells from apoptosis induced by diverse agents (30, 31). This may play a critical role in the apoptotic activity of smoke since BCL-2 exerts its anti-apoptotic effect by maintaining the integrity of mitochondria and preventing the release of pro-apoptotic molecules from its intermembrane space (30, 31). In addition, smoke induced the activation of caspase-8, caspase-9, and executioner caspase-3. Similar effects on caspase activation and BCL-2 expression have been observed with *A. capillaries* smoke on myelogenous leukemia (HL-60) cells (32).

The traditional approach for the introduction of new agents into cancer therapy has been to add the new drug to

accepted and/or established treatment regimens. In this study, we have shown that *A. princeps* smoke reduced the IC_{50} value of ADR 2.7-fold. This would suggest that smoke may decrease the toxicity associated with doxorubicin therapy and that their combination may provide a less toxic, inexpensive and effective cancer chemotherapy.

In summary, human breast cancer cells undergo apoptosis when exposed *in vitro* to a crude preparation of *A. princeps orientalis* smoke and soluble extracts. These preparations induce apoptosis in human breast cancer cells *via* the mitochondrial pathway by causing membrane depolarization and down-regulation of BCL-2.

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Received May 22, 2007
Revised August 2, 2007
Accepted September 10, 2007