*Food Sci. Biotechnol. Vol. 16, No. 4, pp. 531 ~ 536 (2007)*

Food Science and Biotechnology

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# The Essential Oil of *Artemisia iwayomogi* Kitamura Induces Apoptosis on Human Oral Epidermoid Carcinoma Cells

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**Abstract** The aerial part of *Artemisia iwayomogi* Kitamura has traditionally been used for inflammation, infectious disease, cancer, pyretic, diuretic, liver protective effect, and choleretic purposes in Korea. We investigated that the essential oil induces apoptosis in KB cell as evidenced by Hoechst-33258 dye staining, flow cytometry (cell cycles), and DNA fragmentation for nuclear condensation and Western blotting for activation of caspases-3, -8, -9, Bax, Bcl-2, cytochrome *c*, and poly (ADP-ribose) polymerase (PARP) cleavage. In the present study, we found that the essential oil could induce apoptosis in KB cells, as characterized by DNA fragmentation, activation of caspase-3, -8, and -9, and PARP cleavage. The efficacious induction of apoptosis was observed as a dose-dependent. The essential oil-induced apoptotic cell death was accompanied by up-regulation of Bax and down-regulation of Bcl-2. The essential oil also caused the loss of mitochondrial membrane potential and cytochrome *c* release from mitochondria to cytosol. These findings indicate that mitochondrial pathways might be involved in the essential oil-induced apoptosis and enhance our understanding of the anticancer function of the essential oil in herbal medicine.

**Keywords:** *Artemisia iwayomogi*, essential oil, apoptosis, cell cycle, caspases, poly (ADP-ribose) polymerase (PARP), mitochondrial pathway

## Introduction

Most *Artemisia* plants have been used in traditional biomedicine for intestinal bacteria, as food, and for many other purposes in Korea (1). The genus *Artemisia*, one of the largest genera belonging to the compositae family consisting of more than 350 species, is predominantly distributed in the world (2, 3). *Artemisia* species are frequently utilized for the treatment of diseases such as inflammation, hepatitis, cancer and infections by malaria, fungi, bacteria, and viruses (4-8). *Artemisia iwayomogi* Kitamura (*A. messer-schmidtiana* var. *viridis* Besser, Compositae), locally known as *haninjin* or *dowijigi*, is a perennial herb easily found in Korea (1, 9). The aerial part of *A. iwayomogi* has traditionally been used for anti-tumour, immunomodulating, antimutagenic, antioxidant, antibacterial, antifungal, liver protective effect, and choleretic purposes in Korea (10-13).

Apoptosis, distinct from necrosis, is an active process of cell destruction with specific defining biochemical and morphological features characterized by activation of endogenous proteases (caspase) and endonuclease, nuclear/chromatin condensation, internucleosomal cleavage of DNA (DNA ladder), cell shrinkage, dilated endoplasmic reticulum, membrane blebbing, and formation of apoptotic body (14-17). Signals leading to the activation of a family of intracellular cysteine proteases, the caspases, (cysteinyl-aspartate-specific proteinases) play a pivotal role in the initiation and execution of apoptosis induced by various stimuli (18, 19). First-group initiator caspases (caspases-2, -8, -9, -10, and, probably, -11) activate the second-group of caspases (caspases-3, - 6, and -7) (20, 21). In animal cells, PARP is identified as an important substrate of caspase-3, an apoptosis executor. Caspase-3 activity is measured by the cleavage of PARP from a 116-kDa protein to an 85-kDa fragment (22). Many apoptotic stimuli that induce metabolic stress in cell organelles will eventually converge on the mitochondria/apoptosome death pathway (23, 24). The release of cytochrome *c* from mitochondria into the cytosol is central for induction of the intrinsic apoptotic pathway (25, 26). The mitochondrial pathway is regulated by the Bcl-2 family proteins, including anti-apoptoic proteins such as Bcl-2 and Bcl-XL and pro-apoptotic proteins such as Bax, Bad, Bak, and Bid (27-29). Cytosolic proapoptotic protein Bid can be cleaved and activated by active caspase-8 (30, 31). The truncated Bid translocates to the mitochondria and then induces cytochrome *c* release (31). Cytochrome *c* release and caspase activation are mediated by the translocation of cytosolic Bax to the mitochondria in response to the various apoptotic stimuli (32, 33).

In this study, we demonstrated that the essential oil of *A. iwayomogi* induces apoptosis in KB cells via morphology, DNA fragmentation, cell cycle, caspases cascade, inducing cytchrome *c* release from the mitochondria, Bax and Bcl-2 expression, and cleavage of PARP. We found that the

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Received December 3, 2006; accepted May 26, 2007

essential oil induces apoptosis in KB cells through Bcl-2-mediated mitochondrial pathways.

## Matherials and Methods

**Plant material and isolation of the essential oil** The aerial parts of *A. iwayomogi* were collected in September, 2002 from the area of around Paldang Fall of Yanggu-gun in Korea. The identity was confirmed by Dr. Bong-Seop Kil, College of Natural Science, Wonkwang University. The voucher specimen (No-02-01-MR) was deposited at the Herbarium of the College of Natural Science, Wonkwang University. The crushed materials of *A. iwayomogi* (1 kg) were subjected to steam distillation for 3 hr, using a modified Clevenger-type apparatus in order to obtain essential oil. Overall yield was about 0.75%. The essential oil was stored on deep freezer (-70°C) to minimize the loss of volatile compounds.

**Cell culture** KB cells, human oral epidermoid carcinoma cell line (ATCC CCL-17; American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin and 10 μg/mL streptomycin. KB cells were maintained as monolayers in plastic culture plate at 37°C in a humidified atmosphere containing 5% $CO_2$. Then cells were cultured with essential oil at concentrations ranging from 0.05-0.7 mg/mL for 12 hr.

**Cell viability** Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) staining as described (34). Briefly, KB cells were plated at a density of $10^5$ cells/well into 24-well plates and treated with different concentrations of the essential oil for various times (6, 12, 18, 24, and 36 hr). MTT solution (5 mg/mL) was then added to each well incubated for another 4 hr. The purple crystals, produced from the reduction of MTT by metabolically active cells, were solubilized by dimethyl sulfoxide (DMSO; Sigma). After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 540 nm.

**Cell cycle analysis** Flow cytometric analysis was performed as previously described (35). KB cells treated with different concentrations of the essential oil for 12 hr were collected and fixed with 70% ethanol at 4°C for 1 hr. After washing with PBS, the cells were treated with RNase A (Sigma, 1 mg/mL) and stained with propidium iodide (Sigma, 50 μg/mL) for 30 min at 4°C in the dark. The stained cells were quantitatively analyzed by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NH, USA).

**Nuclear morphology analysis** Morphological changes of apoptotic cells were determined by fluorescence microscopy as previously described (36). KB cells treated with different concentrations of the essential oil for 12 hr were collected and fixed in 100% ethanol, and stained with Hoechst-33258 (Sigma, 0.5 μg/mL) for 15 min at 37°C, then visualized under a fluorescence microscope (BX50; Olympus, Tokyo, Japan) with UV excitation at 300-500 nm. The cells with nuclei containing condensed chromatin or with fragmented nuclei were defined as apoptotic cells.

**DNA extraction and DNA gel electrophoresis** The characteristic ladder pattern of DNA break was analyzed by agarose gel electrophoresis (37). KB cells treated with different concentrations of the essential oil for various times were collected, washed with PBS twice, and DNA from KB cells was isolated by a Wizard Genomic DNA purification kit (Promega Co., Madison, WI, USA). Isolated genomic DNA was subjected to 2.0% agarose gel electrophoresis at 100 V for 1 hr. DNA was visualized by staining with ethidium bromide under UV light.

**Preparation of cytosolic and mitochondrial extract** The subcellular fractions were prepared as described previously (38). The harvested pellets were suspended in 5 vol of buffer A [20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol (DTT; Sigma), 1 μg/mL aprotinin, 100 μg/mL phenylmethyl-sulfonylfluoride (PMSF; Sigma), and 250 mM sucrose]. After incubate on ice for 10 min, homogenize cells in an ice-cold dounce tissue grinder (45 strokes) until 70-80% of the nuclei did not have the shiny ring and centrifuge at 700×g for 10 min at 4°C. The supernatant was collected and further centrifuged at 10,000×g for 30 min at 4°C to isolate cytosolin fraction. Cytosolic fraction was stored at -80°C until ready for Western blotting.

**Western blot analysis** Cellular proteins were extracted from control and the essential oil-treated KB cells. The protein concentration was determined using the bicinchoninic acid assay with BSA as standard. Proteins (30 μg) in the cell lysates were separated on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for PARP detection and 12.5% SDS-PAGE for caspsase -3, -8, -9, Bax, Bcl-2, and cytochrome *c* and transferred to immobilon polyvinylidenedifluoride membranes (Millipore Co., Bedford, MA, USA). The membrane was blocked with 1% BSA in PBS-tween 20 for 1 hr at room temperature and incubated with anti-PARP (Oncogene, Darmstadt, Germany, 1:1,000), anti-caspase-3 (Oncogene), -8 (Santa Cruz, CA, USA), -9 (Santa Cruz, 1:1,000), anti-Bax (Santa Cruz, 1:500), anti-Bcl-2 (Santa Cruz, 1:500), and cytochrome *c* (Santa Cruz, 1:1,000) monoclonal antibodies. After washing in PBS-tween 20 three times, the blot was incubated with secondary antibodies and bound antibodies were detected by ECL kit (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, England) with a chemiluminescence, exposing blots to Hyperfilm (Amersham Pharmacia Biotech).

**Statistical analysis** Data are presented as the mean and SEM for the indicated number of separate experiments. Statistical analysis of data was performed using Duncan's multiple range test with one-way analysis of variance. The

level of significance was set at $p$-values less than 0.01.

## Results and Discussion

Several studies focused on developing effective anticancer and chemopreventive approaches have examined the use of essential oils as a natural bioactive substance that can induce sensitive growth inhibition and apoptosis in cancer cells (6, 39). The essential oil of *A. iwayomogi* is a highly purified volatile extract that contains a large number of aromatic components (12). In this study, we examined whether or not the essential oil of *A. iwayomogi* has potential in a cancer chemoprevention using KB cells. KB cells were treated with the essential oil at different concentrations for various times, and the cell viability was determined as described above by MTT assay. As shown in Fig. 1, essential oil inhibited the growth of KB cells in a dose-dependent manner with a range from 0.05 to 0.5 mg/mL and time-dependent manner ($p$<0.01). The results showed the essential oil induces rapid cell death in KB cells. The redistribution of cell cycle phases was analyzed after the treatment with different concentrations of essential oil for 12 hr. The proportion of cells in G0/G1 and S-phase was increased and in G2/M-phase was decreased in essential oil-treated cells when compared with control (Fig. 2). The cells with a sub-G1 DNA content, a hallmark of apoptosis, were seen in a dose-dependent manner in essential oil-treated cells. Nucleic acid staining with Hoechst 33342 revealed typical apoptotic nuclei, which exhibited highly fluorescent condensed chromatin in cells treated with different concentrations of the essential oil for 12 hr (Fig. 3). The morphological changes and cell death of KB cells were significantly increased at 0.5 mg/mL essential oil, and most cells were detached from the dishes and cell rounding and shrinking occurred at the essential oil. To determine whether the essential oil, induces apoptosis in KB cells, we investigated the DNA fragmentation, which is a biochemical hallmark for apoptosis. The results

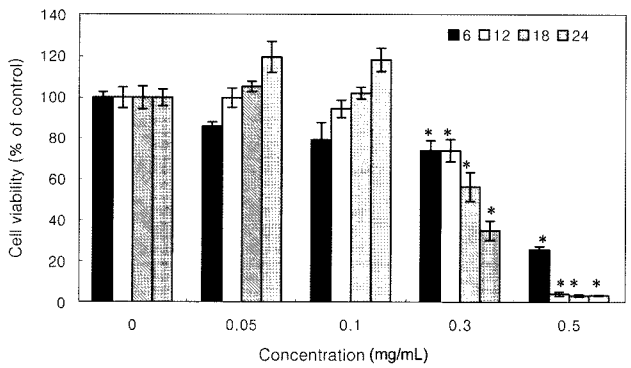


**Fig. 1. Effect of the essential oil extracted from *Artemisia iwayomogi* on cell proliferation in KB cells.** KB cells were plated into 24 well plates and treated with different concentrations of the essential oil for various times. Cell proliferation was determined by the MTT assay and expressed as percentage of the absorbance value obtained without essential oil. The results are expressed as the mean±SD from 3 different experiments with triplicate cultures. *$p$<0.01 compared with control.

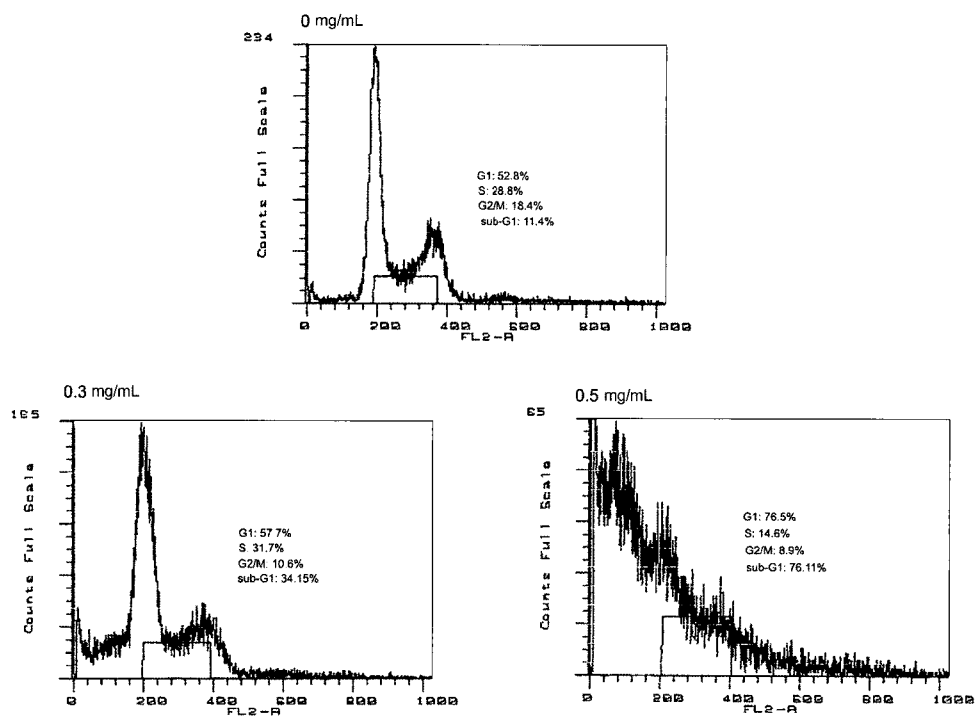


**Fig. 2. *Artemisia iwayomogi* essential oil-induced apoptosis in KB cells.** KB cells were treated with 0.3 and 0.5 mg/mL essential oil for 12 hr. Non-treated cells were used as a negative control (0 mg/mL). Following the treatment, cells were collected for 3 kinds of experiments for apoptosis induction. The percentages of apoptotic cells were determined by propidium iodide staining followed by flow cytometric analysis.

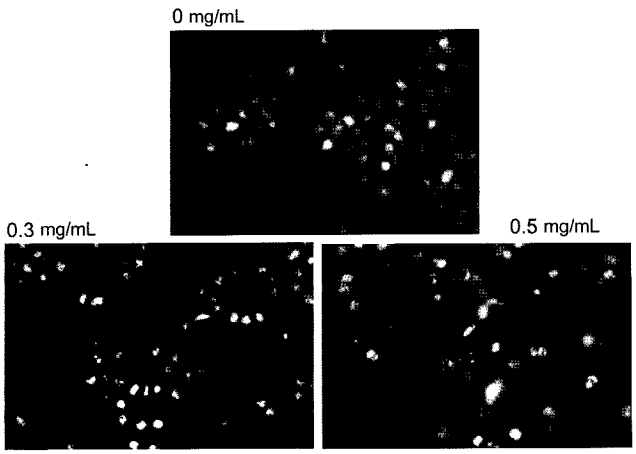


**Fig. 3. *Artemisia iwayomogi* essential oil-induced apoptosis in KB cells.** KB cells were treated with the essential oil for 12 hr. Non-treated cells were used as a negative control (0 mg/mL). Following the treatment, cells were collected for 3 kinds of experiments for apoptosis induction. The morphologic change analysis under fluorescence microscope after staining with Hoechst 33258. The apoptotic cells are indicated with arrows. Normal nuclear morphology was observed in untreated cells; in contrast, small, fragmented, and condensed nuclei with typical apoptotic morphology were observed in treated cells.

demonstrated that the essential oil induced endonucleolytic DNA cleavage in a time- and dose-dependent manner (Fig. 4). The essential oil-induced cell death was accompanied by nuclear condensation and DNA fragmentation characteristic of apoptosis. And also, we observed that the KB cells treated with the essential oil cause morphologic changes such as apoptotic bodies and chromatin condensation as well as DNA fragmentation into oligonucleosomal-sized fragments. Apoptosis-associated nuclear condensation

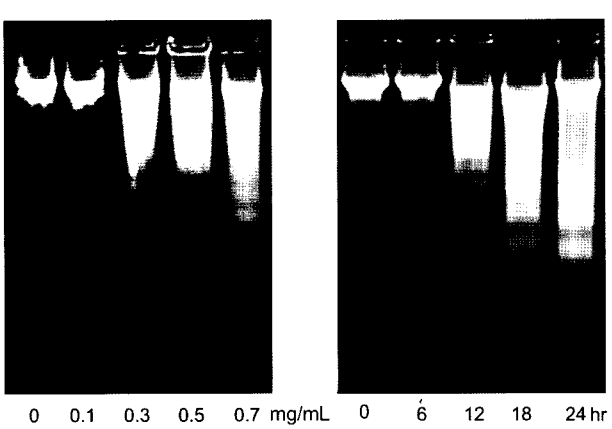


**Fig. 4. *Artemisia iwayomogi* essential oil-induced apoptosis in KB cells.** KB cells were treated with different concentrations of the essential oil for various times. KB cells were exposed to the indicated concentrations of the essential oil for 12 hr and to 0.5 mg/mL for the indicated times. Cells were harvested by centrifugation and DNA was extracted. The DNA fragments were separated on 2.0% agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide.

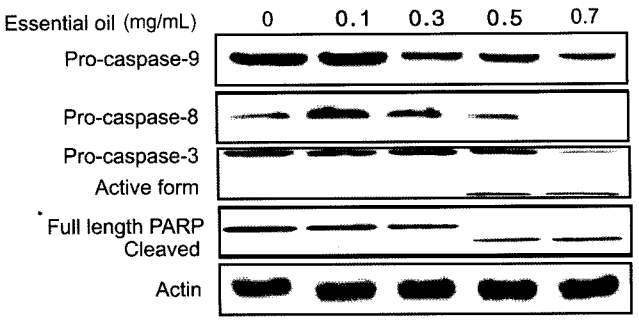


**Fig. 5. *Artemisia iwayomogi* essential oil-induced apoptosis involves activation of caspase-3, -8, -9, and PARP cleavage.** KB cells were treated with different concentrations of the essential oil for 12 hr. Proteins in whole cellular lysates with untreated control were electrophoresed in SDS-PAGE gel and transferred to nitrocellulose membrane. Caspase-3, -8, -9, and PARP were identified using specific antibody. Activation of caspase-3, -8, -9, and PARP cleavage were increased as a dose-dependent manner.

is usually accompanied by oligonucleosomal DNA fragmentation into oligomers of 180 base pairs (23).

Apoptosis is a tightly regulated progress under the control of several signaling pathways, such as caspase and mitochondrial pathways (18, 24, 25, 30). Cytochrome *c* release from mitochondrion to cytosol causes caspase-9-dependent activation of caspase-3 and cleavage of the DNA reparatory protein PARP (31, 40). We investigated that the essential oil induces activation of these caspases. KB cells were treated with different concentrations of the essential oil for 12 hr, and processing of pro-caspases-3, -8, and -9 was monitored by Western blotting (Fig. 5). At concentrations more than 0.1 mg/mL, the essential oil induced processing of pro-caspase-3 into active forms, processing of pro-caspase-9 into active forms and processing of pro-caspase-8 into active forms within 12 hr. To confirm further the apoptosis induced by the essential oil, we investigated the cleavage of PARP in KB-treated cells. Treatment of KB cells with 0.5 mg/mL essential oil caused a proteolytic cleavage of PARP, with accumulation of the characteristic 85-kDa fragments and a concomitant disappearance of the full-length 116-kDa protein (Fig. 5). The cytosol levels of cytochrome *c* were examined using western blot analysis. The results of this analysis showed the concentration-response effect of the essential oil on cytochrome *c* translocation after 12 hr treatment was also determined (Fig. 6), revealing that the essential oil increased translocation of cytochrome *c* from the mitochondria to the cytosol in a dose-dependent manner. In this study, we found that the essential oil induced the release of cytochrome *c* and the activation of caspase-3, -8, and -9. Overexpression of Bcl-2 or Bcl-XL can protect against chemotherapy-induced release of mitochondrial cytochrome *c*, caspase activation, and DNA fragmentation (30, 41). We checked the Bax truncation and Bcl-2 expression in essential oil-treated KB cells. Figure 6 was shown a dose-response truncation of Bax protein and reduction of Bcl-2 protein in essential oil-treated KB cells. These results suggest that the essential oil might induce apoptosis through Bax- and Bcl-2-mediated mitochondrial pathway. In the present study, the increase in the essential

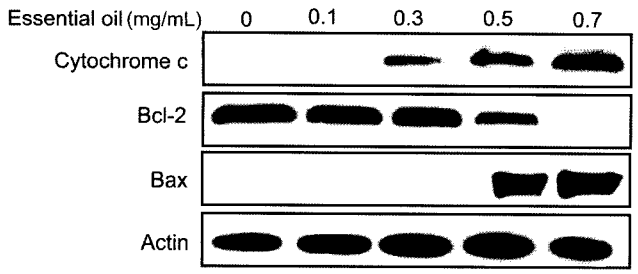


**Fig. 6. *Artemisia iwayomogi* essential oil-induced apoptosis involves mitochondrial dysfunction pathway.** KB cells were treated with different concentrations of the essential oil for 12 hr. Cytochrome *c* was detected by Western blotting analysis with cytochrome *c* antibody. Whole-cell lysate was used for Western blotting analysis with Bcl-2 and Bax antibody, respectively. Cytochrome *c* release, Bcl-2 reduction, and Bax truncation were increased as a dose-dependent manner.

oil-induced apoptosis was associated with an increase in levels of Bax protein, which heterodimerizes with and thereby inhibits Bcl-2. Our study demonstrated that the essential oil alters the ratio of Bcl-2 and Bax and, therefore, lead to the apoptosis of KB cells. These findings indicate that mitochondrial pathways might be involved in the essential oil-induced apoptosis on a dose-dependent manner with a range from 0.05 to 0.5 mg/mL and enhance our understanding of the anticancer function with high dose of the essential oil in herbal medicine.

In conclusion, these results also indicate the possibility of exploitation of the essential oil of *A. iwayomogi* as induction of apoptosis in KB cells. Induction of cancer cell apoptosis or death, without affecting healthy cells or producing side effects, is a major goal for development of new therapeutic agents. The normal level of essential oil of *A. iwayomogi* used in foods has already been established for its safety. These findings suggest that the essential oil of *A. iwayomogi* may have potential use as cancer chemopreventive and therapeutic agent.

## Acknowledgments

This paper was supported by Wonkwang University in 2005.

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