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# Growth inhibition and induction of apoptosis in MCF-7 breast cancer cells by *Antrodia camphorata*

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**Abstract**

*Antrodia camphorata* (*A. camphorata*) is well known in Taiwan as a traditional Chinese medicine, and it has been shown to exhibit antioxidant and anticancer effects. In this study, therefore, its ability to induce apoptosis in cultured MCF-7 breast cancer cells was studied. Treatment of the MCF-7 cells with a variety of concentrations of the fermented culture broth of *A. camphorata* (25–150 μg/ml) resulted in dose- and time-dependent sequences of events marked by apoptosis, as shown by loss of cell viability, chromatin condensation, internucleosomal DNA fragmentation, and sub-G1 phase accumulation. Furthermore, apoptosis in the MCF-7 cells was accompanied by the release of cytochrome *c*, activation of caspase 3, and specific proteolytic cleavage of poly (ADP-ribose) polymerase (PARP). Although, the *A. camphorata*-induced apoptosis was associated with Bax protein levels, negligible Bcl-2 reduction was observed. Interestingly, *A. camphorata* induced dose-dependent reactive oxygen species (ROS) generation in MCF-7 cells. Analysis of the data suggests that *A. camphorata* exerts antiproliferative action and growth inhibition on MCF-7 cells through apoptosis induction, and that it may have anticancer properties valuable for application in drug products.


*Keywords:* *Antrodia camphorata*; MCF-7 cells; Apoptosis

## 1. Introduction

A new basidiomycete, *Antrodia camphorata*, in the Polyporaceae (Aphyllophorales), which causes brown heart rot in *Cinnamomun kanehirai* hay (Lauraceae) in Taiwan, has been identified as a new genus of the *Antrodia* species [1,2]. *A. camphorata* is rare and expensive as it grows only on the inner heart-wood wall of the *C. kanehirai* and cannot be cultivated. It has been utilized in traditional Chinese medicine for the treatment of food and drug intoxication, diarrhea, abdominal pain, hypertension, skin itches, and liver

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cancer [3], however, very few biological activity tests are reported.

Recently, the relationship between apoptosis and cancer has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression and metastasis involve the alteration of normal apoptotic pathways [4]. Apoptosis provides a number of clues with respect to effective anticancer therapy, and many chemotherapeutic agents reportedly exert their antitumor effects by inducing apoptosis in cancer cells [5]. Apoptosis is a strictly regulated pathway responsible for the ordered removal of superfluous, aged, and damaged cells. It not only plays an important role in the development and maintenance of tissue homeostasis, but it also represents an effective mechanism by which harmful cells can be eliminated [6,7]. Morphological hallmarks of this process includes loss of cell volume, hyperactivity of the plasma membrane, and condensation of peripheral heterochromatin, followed by cleavage of the nucleus and cytoplasm into multiple membrane-enclosed bodies containing chromatin fragments [8–11]. Recently, considerable attention has been devoted to the sequence of events referred to as apoptotic cell death and the role of this process in mediation of the lethal effects of the diverse antineoplastic agents.

For 2000 years, medicinal mushrooms have been used in China to improve health and achieve longevity. These mushrooms reportedly possess antitumour and immunomodulating activities [12,13]. *A. camphorata* has recently become popular as a remedy for drug in Taiwan, as well as a source of physiologically beneficial mushrooms. In our previous study, *A. camphorata* was used for the inhibition of AAPH-induced oxidative hemolysis and lipid/protein peroxidation of normal human erythrocytes [14]. Interestingly, *A. camphorata* exhibits significant apoptotic cell death against leukemia HL-60 cells, but not against cultured human endothelial cells [15]. In another study, an association between the antioxidant activity of *A. camphorata* and its polyphenol, triterpenoid, and polysaccharide contents was demonstrated, based on evaluations of different antioxidant test systems [16]. Scientific interest in these active compounds (polysaccharides, triterpenoids, and polyphenols isolated from mushrooms) has recently been aroused due to the antiinflammatory, antimutagenic, and anticarcinogenic properties [17–22].

Breast cancer is the most common malignancy in American and Northwestern European women. Approximately one-third of the women with breast cancer developed metastases and ultimately died of the disease. In 1970, the estrogen receptor-positive MCF-7 cell line was derived from a patient with metastatic breast cancer [23]. Since then MCF-7 cell has become a prominent model system for the study of breast cancer as it relates to the susceptibility of the cells to apoptosis. Despite the fact that many tumors initially respond to chemotherapy, breast cancer cells can subsequently survive and gain resistance to the treatment [24]. Further, it has become increasingly important in the treatment of a number of major solid tumors, particularly metastatic and drug-resistant breast cancers [25]. In this study, the effects of the fermented broth of *A. camphorata* (harvested from submerged cultures) on cultured MCF-7 human breast cancer cells was investigated due to the interesting biological activities reported and their potential clinical application. The data reported herein appear to demonstrate that *A. camphorata* induces massive death in the MCF-7 cells, the dying cells exhibiting the ultrastructural and biochemical features that characterize apoptosis. Additionally, the biochemical steps linking *A. camphorata* to the apoptotic process in these cells were investigated.

## 2. Materials and methods

### 2.1. Chemicals

Fetal bovine serum (FBS), DMEM, penicillin–streptomycin (PS) and glutamine (GIBCO Laboratories, Grand Island, NY), rabbit polyclonal antibody against Bcl-2, Bax, cytochrome *c*, and caspase 3 (Santa Cruz Biotechnology, Inc., Heidelberg, Germany), PARP rabbit polyclonal antibody (Upstate biotechnology, Lake Placid, NY), mouse monoclonal antibody against actin (Sigma Chemical Co., St Louis, MO), the enhanced chemiluminescence kit (ECL; Pierce, Rockford, IL) and caspase 3 substrates (Promega, Madison, Wis) were obtained from various suppliers. All other chemicals were of the highest

grade commercially available and supplied either by Merck or Sigma.

### 2.2. Preparation of fermented culture broth of A. camphorata

Culture of *A. camphorata* was inoculated on potato dextrose agar and incubated at 30 °C for 15–20 days. The whole colony was then cut and placed into the flask with 50 ml sterile water. After homogenization, the fragmented mycelia suspension was used as the inoculum. The seed culture was prepared in a 20 l fermentor (BioTop) agitated at 150 rpm at an aeration rate of 0.2 vvm at 30 °C. A 5-day culture of 15 l mycelia inoculum was inoculated into a 250 l agitated fermentor (BioTop). The fermentation conditions were the same as for the seed fermentation but an aeration rate of 0.075 vvm was used. The fermentation product was then harvested at the 331st hour and poured through the non-woven fabric on a 20-mesh sieve to separate the deep red fermented culture broth and mycelia, and then centrifuged at $3000 \times g$ for 10 min, followed by passage through a 0.2 μm filter. The culture broth was concentrated under vacuum and freeze-dried to powder form. The yield of dry matter from the culture broth was approximately 9.72 g/l. For preparation of the aqueous solution, the powder samples were solubilized with 10 mM sodium phosphate buffer (pH 7.4), containing 0.15 M sodium chloride (PBS) at 25 °C. The stock solution was stored at −20 °C before analysis for apoptotic properties.

### 2.3. Cell culture and assessment of cell growth and viability

The human breast cancer cell line, MCF-7, and the human healthy breast cell line, HBL100, were obtained from the American type Culture Collection (Rockville, MD) and the European Collection of Animal Cell Cultures, respectively. These cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 1% penicillin–streptomycin–neomycin in a humidified incubator (5% $CO_2$ in air at 37 °C). Cells were seeded in six-well plates prior to *A. camphorata* addition. The MCF-7 cells were incubated with *A. camphorata* at various concentrations (0, 25, 50, 100, and 150 μg/ml) for 24, 48 and 72 h. Cultures were harvested and monitored for cell number by counting cell suspensions with a hemocytometer. Cell growth ($2.0 \times 10^5$ cells/well) and viability ($1.0 \times 10^6$ cells/-well) were checked before and after treatment with *A. camphorata* using trypan blue exclusion and examined using phase contrast microscopy.

### 2.4. DNA gel electrophoresis (DNA laddering)

The presence of internucleosomal DNA cleavage in MCF-7 cells ($2.0 \times 10^6$ cells/60-mm dish) was investigated using DNA gel electrophoresis. The DNA purification kit (Gentra Corp., Minneapolis, MN, USA) was used according to the manufacturer's instructions. DNA purity and concentration were determined by electrophoresis on a 1.5% agarose gel containing ethidium bromide, followed by observation under ultraviolet illumination.

### 2.5. Flow cytometric analysis of apoptosis

Cellular DNA content was determined by flow cytometric analysis of PI-labeled cells. MCF-7 cells were grown to exponential phase, seeded at a density of $2.0 \times 10^6$ cells/60-mm dish, and treated with the indicated concentrations of *A. camphorata* (25–150 μg/ml) for 24 h. Cells were harvested, fixed in ice-cold 70% ethanol, stored at 4 °C, washed with phosphate-buffered saline (pH 7.2), treated with 25 μg/ml RNase A at 37 °C for 15 min, and stained with 50 μg/ml propidium iodide (PI) for 20 min. For flow cytometric analysis, a FACSCalibur flow cytometer (Becton Dickinson, NJ) equipped with a single argon ion laser was used. The excitation wavelength was 488 nm, and the emission filters were 515–545 BP, 572–588 BP, and 600 LP. Forward light scatter, which is correlated with the size of the cell, and right-angle light scatter, which is correlated with the complexity of the cytoplasm, was used to establish size gates and exclude cellular debris from the analysis. DNA content of 10,000 cells per analysis was monitored using the FACSCalibur system. DNA fluorescence of PI-stained cells was evaluated by excitation at 488 nm and monitoring through a 630/22 nm band pass filter. A minimum of 10,000 cells per sample was used for analysis performed using CellQuest software. Apoptotic nuclei were identified as a subploid DNA peak, and were

distinguished from cell debris on the basis of forward light scatter and PI fluorescence. Representative flow cytometry patterns are shown.

### 2.6. Preparation of total cell extract and immunoblot analysis

To prepare the whole-cell extract, MCF-7 cells ($1.0\times10^7$ cells/100-mm dish) were detached and then washed once in cold phosphate-buffered saline (PBS) and suspended in 100 μl lysis buffer (10 mM Tris–HCl (pH 8), 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM DTT, and 1 mM phenylmethyl sulfony flouride). The suspension was put on ice for 20 min and then centrifuged at 5000 rpm for 20 min at 4 °C. Total protein content was determined by Bio-Rad protein assay reagent using bovine serum albumin as the standard, and protein extracts were reconstituted in sample buffer (0.062 M Tris–HCl, 2% SDS, 10% glycerol, and 5% [vol/vol] β-mercaptoethanol), and the mixture boiled for 5 min. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, separated on 10% SDS polyacrylamide gel, followed by transfer of the proteins to PVDF membranes overnight. Membranes were blocked with 0.1% Tween-20 in Tris-buffered saline containing 5% non-fat dry milk for 20 min at room temperature, and the membranes were reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse Ab for 2 h before being developed using the Super-Signal ULTRA chemiluminescence substrate.

### 2.7. Caspase 3 activity assay

Caspase 3 activity in the supernatant was determined using ApoAlert Caspase Colormetric Assay Kits [26]. Briefly, following pre-incubation with *A. camphorata*, cells were counted and centrifuged ($2.0\times10^6$ cells/60-mm dish) at $400\times g$ for 10 min. Then cell pellets were lysed using lysis buffer on ice for 10 min. After centrifugation at 13,000 rpm at 4 °C for 3 min using an Eppendorf centrifuge, supernatants were collected and added with DEVD-pNA to the final concentration of 50 μM. Each sample was incubated at 37 °C for 1 h in a water bath, and the optical density at 405 nm measured. The standard curve was made by measuring the $A_{405}$ of various quantities of pNA, with the $A_{405}$ value then converted to determine the amounts of pNA produced.

### 2.8. ROS generation by chemiluminescence assay

ROS (reactive oxygen species) production was determined according to the method of Lu et al., [27] with some modification. Briefly, MCF-7 cells were suspended in DMEM/10% FBS with or without *A. camphorata* at 37 °C for 24 h. After trypsinization, the cells were washed, resuspended at $2\times10^5$ cells/ml in PBS, and then placed in a dark chamber containing luminol (1 mM), and light emission measured using an ultrasensitive chemiluminescence detector (model CLD-110; Tohoku Electronic Industrial Co., Sendai, Japan) at 10-s intervals for a total of 10 min. The total chemiluminescence intensity was calculated by integrating the area under the curve minus the background level, which was equal to the dark average, and the results expressed as counts per 10 s.

### 2.9. Statistics

Mean data values are presented with their deviation (mean ± SEM). All data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $P<0.05$ for all tests.

## 3. Results

In this study, MCF-7, a human breast cancer cell line, was used to investigate the capability of the fermented broth of *A. camphorata* (harvested from submerged culture) to induce apoptosis, and to elaborate the molecular mechanism(s) involved.

### 3.1. Effect of A. camphorata on cell growth and viability of MCF-7 cells

To investigate the potential effects of *A. camphorata* on proliferation and survival of MCF-7 cells, the cells were exposed to 0–150 μg/ml of *A. camphorata* for 24, 48 and 72 h. Figs. 1 and 2 show that *A. camphorata* induces cell death in a dose- and time-dependent manner, as determined using trypan blue exclusion. Further, exposure to *A. camphorata*

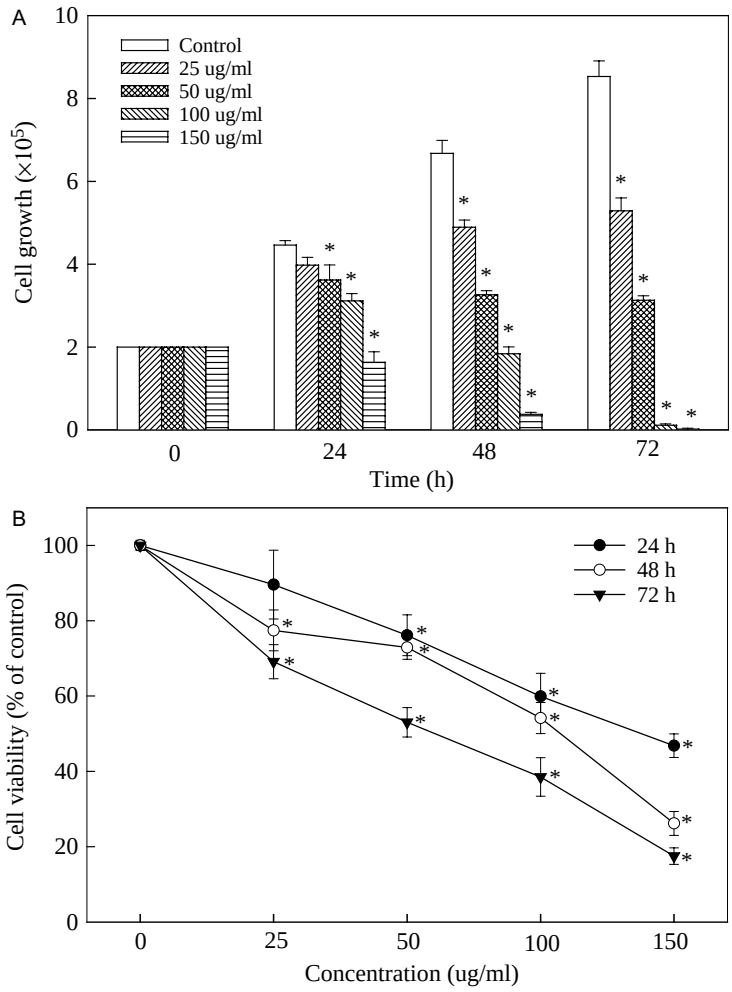


Fig. 1. Effects of *A. camphorata* on MCF-7 cell growth (A) and viability (B). Cells were treated with 0, 25, 50, 100, and 150 μg/ml of *A. camphorata* for 24, 48, and 72 h. Control cells were maintained in the vehicle for the indicated time periods. Results are presented as mean ± SEM of three assays. * indicates significant difference in comparison to control group ($P < 0.05$).

was associated with cell shrinkage as detected in phase-contract micrographs (Fig. 2).

### 3.2. Induction of DNA fragmentation by A. camphorata

Furthermore, agarose-gel electrophoresis of *A. camphorata*-treated chromosomal DNA showed a ladder-like pattern of DNA fragments consisting of multiples of approximately 180–200 base pairs. The apoptosis-inducing activity of *A. camphorata* was dose- and time-dependent (Fig. 3(A) and (B)).

### 3.3. Effect of A. camphorata on cellular DNA content of MCF-7 cells

In addition, the profile of the DNA content was obtained by using flow cytometric analysis to measure the fluorescence of PI binding to DNA (Fig. 4). MCF-7 cells with lower DNA staining relative to diploid analogs were considered apoptotic. It was noted that there was a remarkable accumulation of subploid cells, the so-called sub-G1 peak, in *A. camphorata*-treated MCF-7 cells (Fig. 4(B)–(E)) when compared with the untreated group (Fig. 4(A)).

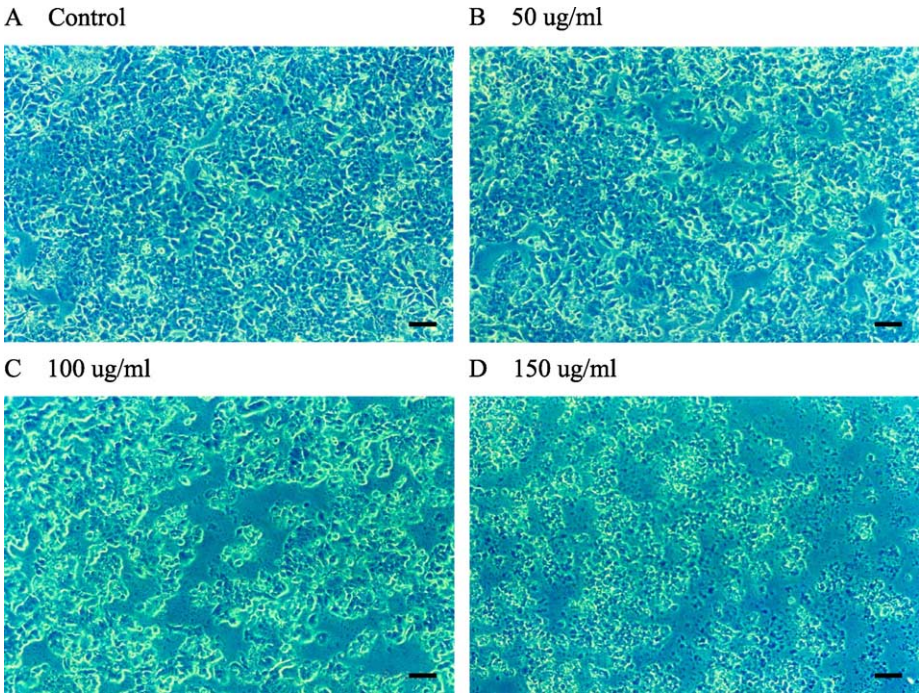


Fig. 2. Phase-contrast micrographs of *A. camphorata*-treated MCF-7 cells. Cells treated with 0, 50, 100, and 150 μg/ml of *A. camphorata* for 24 h. Typical result from three independent experiments is shown. Bar represents 50 μm.

Combined with the phenomena shown in DNA argarose electrophoresis, our findings suggest that the viability reduction observed after *A. camphorata* treatment may result from apoptosis induction in MCF-7 cells.

### *3.4. Effect of A. camphorata on cytochrome c release, caspase 3 activity, and PARP cleavage*

Studies indicate that treatment of cells with a variety of chemotherapeutic agents is accompanied by increased cytosolic translocation of cytochrome *c*, activation of caspase 3, and degradation of PARP [28–30]. In the present study, the cytosol levels of cytochrome *c* were examined using western blot analysis. Our results reveal that *A. camphorata* induces release of cytosolic cytochrome *c* in a dose- and time-dependent manner (Fig. 5), with the amount gradually increasing from 4 h. Since cytochrome *c* is reportedly involved in the activation of the caspases that trigger apoptosis, we investigated the role of caspase 3 in the cell response to *A. camphorata*. Caspase 3 (CPP32) is a cytosolic protein that normally exists as a 32-kDa inactive precursor. It is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis [28]. As shown in Fig. 5, the involvement of caspase 3 activation is further supported by immunoblotting analysis in which *A. camphorata* evidently induces proteolytic cleavage of pro-caspase 3 into its active form, a 17-kDa fragment. Caspase activity in the *A. camphorata*-treated MCF-7 cells was also measured using colormetric assay. As illustrated in Fig. 6, *A. camphorata* induced an increase in caspase 3 activity in treated MCF-7 cells. Since PARP-specific proteolytic cleavage by caspase 3 is considered to be a biochemical characteristic of apoptosis, a western blotting experiment was conducted using the antibody against PARP. PARP is a nuclear enzyme which is involved in DNA repair, and it has been demonstrated that the 116 kDa PARP protein is cleaved into a 85 kDa fragment [28]. Fig. 5 shows that PARP is cleaved into a 85 kDa fragment after the addition of *A. camphorata*.

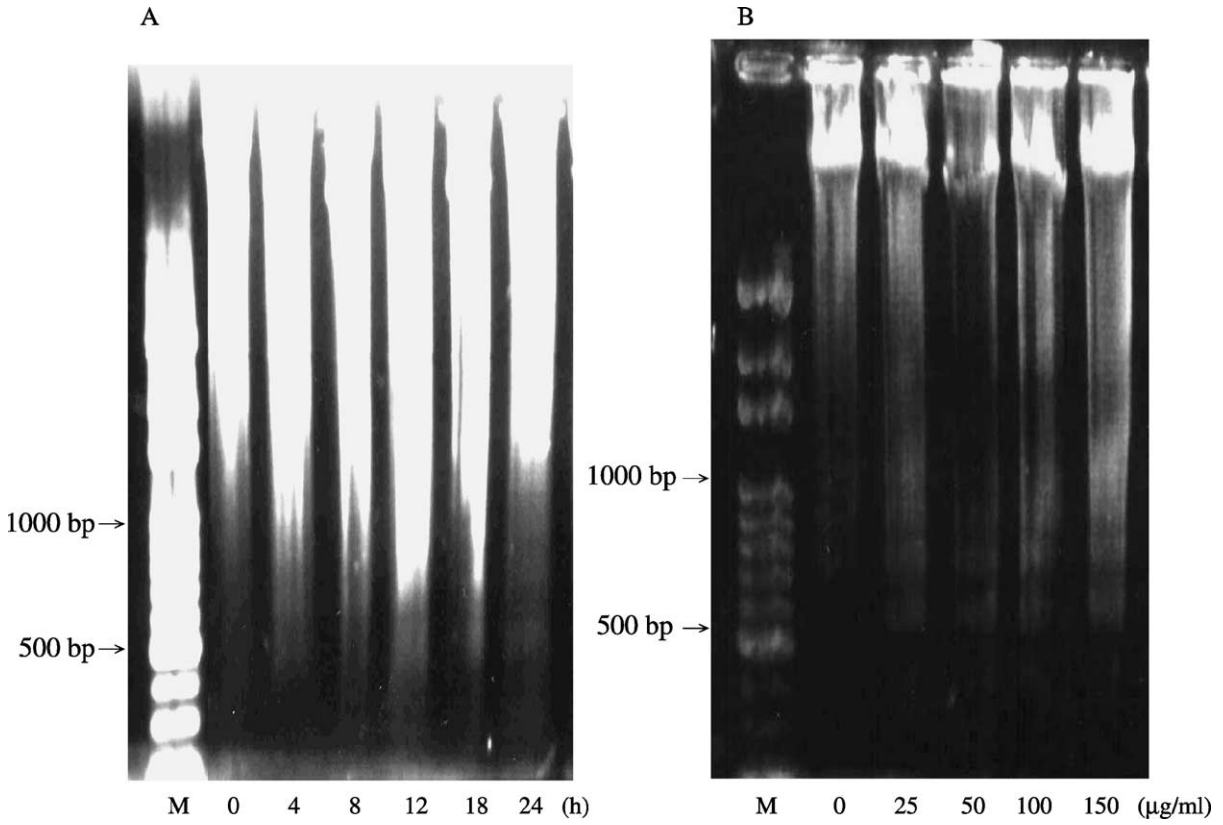


Fig. 3. DNA fragmentation of MCF-7 cells exposed to *A. camphorata*. (A) MCF-7 cells incubated with 150 μg/ml of *A. camphorata* at 0, 4, 8, 12, 18, and 24 h. (B) MCF-7 cells incubated with 0, 25, 50, 100, and 150 μg/ml of *A. camphorata* for 24 h. DNA ladders reflecting the presence of DNA fragments were viewed on ethidium-bromide-stained gel. Typical result from three independent experiments is shown. M, molecular-weight markers.

### *3.5. Effect of A. camphorata on Bcl-2 and Bax protein*

Bcl-2 and Bax protein levels were studied in cultured MCF-7 cells to examine the involvement of Bcl-2 and Bax in *A. camphorata*-mediated apoptosis. Western blot analysis of Bcl-2 and Bax exposed to *A. camphorata* was resolved on 10% SDS-PAGE. As showed in Fig. 7, incubation of MCF-7 cells with *A. camphorata* dramatically increased Bax protein levels in a dose- and time-dependent manner. There was no effect on the Bcl-2 protein, however. These results indicate that *A. camphorata* may disturb the Bcl-2 and Bax ratio.

### *3.6. ROS generation in A. camphorata-treated MCF-7 cells*

The production of ROS can be detected by using an ultrasensitive chemiluminescence analyzer, which monitors the emission of chemiluminescence derived from the energy of a chemical reaction. Luminol is typically used as a chemiluminigenic probe for estimating ROS generation [31,32]. As showed in Fig. 8, the unstimulated MCF-7 (control) showed a basal level of luminol-amplified chemiluminescence. Incubation of MCF-7 cells with *A. camphorata* at concentrations of 25–150 μg/ml caused a significant increase in chemiluminescence response. The results indicate that *A. camphorata* induces ROS generation in MCF-7 cells in a dose-dependent manner.

### *3.7. Effects of A. camphorata on the growth of HBL100 cells*

To test whether *A. camphorata* affects the human healthy breast cells, its effects on the growth of HBL100 cells were also examined. The number of

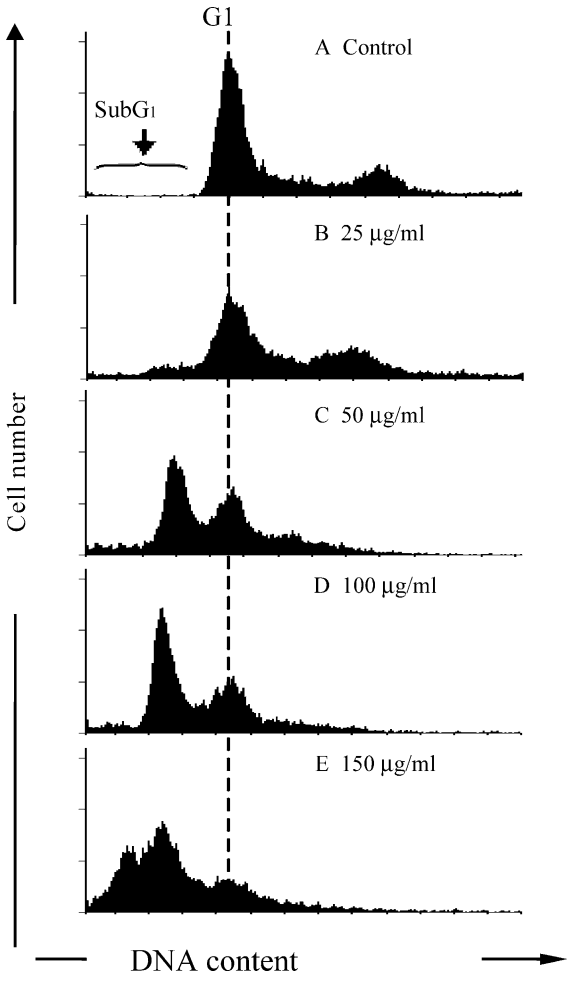


Fig. 4. Effect of *A. camphorata* on the cellular DNA content of MCF-7 cells. Flow cytometric analysis of DNA fragmentation. Cells were grown in the absence (control) or the presence of *A. camphorata* (25–150 μg/ml) for 24 h, stained with propidium iodide, and analyzed by flow cytometry for DNA content. Arrows indicate predicted location of fragmented DNA or Sub-G1 population. Each plot is representative of three similar experiments.

HBL100 cells was not affected by *A. camphorata* at 0, 25, 50, 100 and 150 μg/ml after 24 h of incubation (Fig. 9). There was a decrease in cell number at 48 and 72 h with the higher dose of *A. camphorata*. Experiments were conducted to compare the response of MCF-7 and HBL100 cells to *A. camphorata* treatment. Cell growth dropped in response to *A. camphorata* treatment in both cell lines, but MCF-7 cells were more sensitive than HBL100 cells.

## 4. Discussion

The results reported herein reveal that the fermented culture broth of *A. camphorata* harvested from submerged cultures exerts antiproliferative action and growth inhibition in cultured human breast cancer MCF-7 cells. The dying cells exhibit the ultrastructural and biochemical features that characterize apoptosis, as shown by loss of cell viability, chromatin condensation, internucleosomal DNA fragmentation, and sub-G1 phase accumulation. It was also determined that cytotoxicity of the culture medium is lower than that of *A. camphorata* in submerged culture (data not shown), indicating that the cytotoxic components of *A. camphorata* must be derived from secondary metabolites of the mycelia. In this study, the data showed that the apoptosis-inducing (cytotoxic) activity of *A. camphorata* was specific to estrogen receptor-positive breast cancer lines, MCF-7, since no such strong action was detected at the level of the estrogen receptor-negative healthy breast cell line, HBL100. The effects were also observed in human premylocytic leukemia HL-60 cells, but were not found in healthy erythrocytes or human umbilical vein endothelial cells (HUVECs) [15]. Altogether, we suggest that *A. camphorata* specifically affected the cell growth and induced apoptotic cell death in cancer cell lines.

This study also defines those events, most of which are used as biomarkers of apoptosis, that were associated with *A. camphorata*-induced MCF-7 human breast apoptotic cell death. Cells undergoing apoptosis were found to have an elevation of cytochrome *c* in the cytosol, with a corresponding decrease in the mitochondria [33]. After the release of mitochondrial cytochrome *c*, the cysteine protease 32 kDa proenzyme CPP32, a caspase 3, is activated by proteolytic cleavage into an active heterodimer [28]. Activated caspase 3 is responsible for the proteolytic degradation of poly (ADP-ribose) polymerase, which occurs at the onset of apoptosis [29,30]. In this study, we produced evidence demonstrating that *A. camphorata*-induced apoptosis of MCF-7 cells is mediated by increased cytosolic translocation of cytochrome *c*, activation of caspase 3, and degradation of PARP. Recently, numerous papers have reported that internucleosomal DNA fragmentation is not essential for apoptotic cell death, and that some

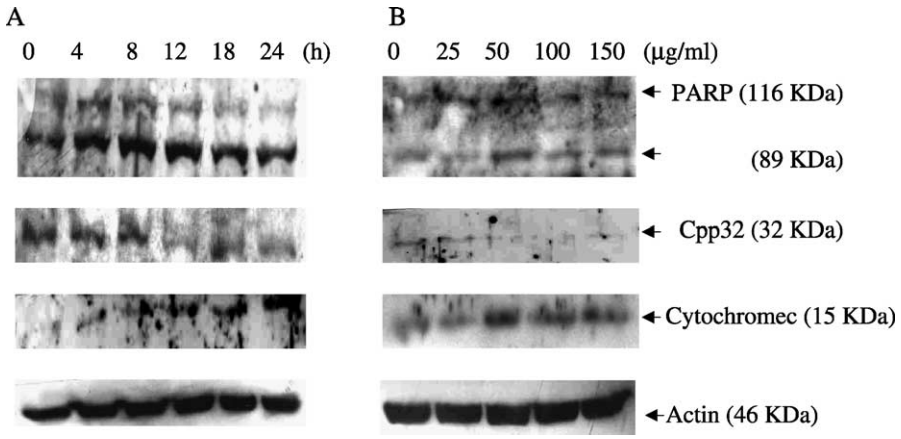


Fig. 5. Western blot analysis of cytochrome *c*, caspase 3, and PARP protein levels exposed to *A. camphorata*. (A) MCF-7 cells treated with 150 μg/ml of *A. camphorata* at 0, 4, 8, 12, 18, and 24 h. (B) MCF-7 cells treated with 0, 25, 50, 100, and 150 μg/ml of *A. camphorata* for 24 h. Protein (50 μg) from each sample was resolved on 10% SDS-PAGE, and western blot performed. Typical result from three independent experiments is shown.

necrotic cell death is accompanied by internucleosomal DNA fragmentation, suggesting the possibility that this fragmentation may not be sufficient as an indicator of apoptotic cell death [34,35]. It is clear, however, that the central mechanism of apoptosis is evolutionarily conserved, and that caspase activation is an essential step in this complex apoptotic pathway [36]. The presented data, therefore, provides more important evidence that *A. camphorata* -induced MCF-7 cell death is apoptosis.

Recently, it has been shown that the Bcl-2 family plays an important regulatory role in apoptosis, either as activator (Bax) or as inhibitor (Bcl-2) [37–39]. It has also been demonstrated that the gene products of Bcl-2 and Bax play important roles in apoptotic cell death [40–42]. Of the Bcl-2 family members, the Bcl-2 and Bax protein ratio has been recognized as a key factor in regulation of the apoptotic process [37–39]. In the present study, the increase in *A. camphorata*-induced apoptosis was associated with an increase in levels of Bax protein, which heterodimerizes with, and thereby inhibits, Bcl-2. Negligible Bcl-2 reduction was observed, however. Analysis of our data indicates that *A. camphorata* may disturb the Bcl-2/Bax ratio and, therefore, lead to apoptosis of MCF-7 cells.

Many of the agents that induce apoptosis are oxidants or stimulators of cellular oxidative metabolism, while many inhibitors of apoptosis show antioxidant activity [43]. Indeed, factors for oxidative stress, such as ROS production [44–48], lipid peroxidation [49], downregulation of the antioxidant defenses characterized by reduced glutathione levels [50], and reduced transcription of superoxide dismutase, catalase, and thioredoxin, have been observed in some apoptotic processes [51]. Moreover, ROS can

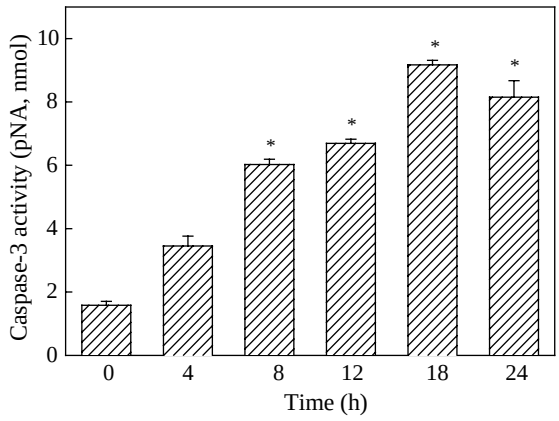


Fig. 6. Time course for *A. camphorata*-induced caspase 3 activation of MCF-7 cells. Cells were treated with 150 μg/ml of *A. camphorata*, with samples harvested at 0, 4, 8, 12, 18, and 24 h. Enzyme activity of the caspase proteases was determined as described in Section 2. The release of pNA was measured at 405 nm using a spectrophotometer. Caspase activity was expressed as pNA change (nmol). Results are mean ± SEM of three assays. * indicates significant difference in comparison to control group ($P < 0.05$).

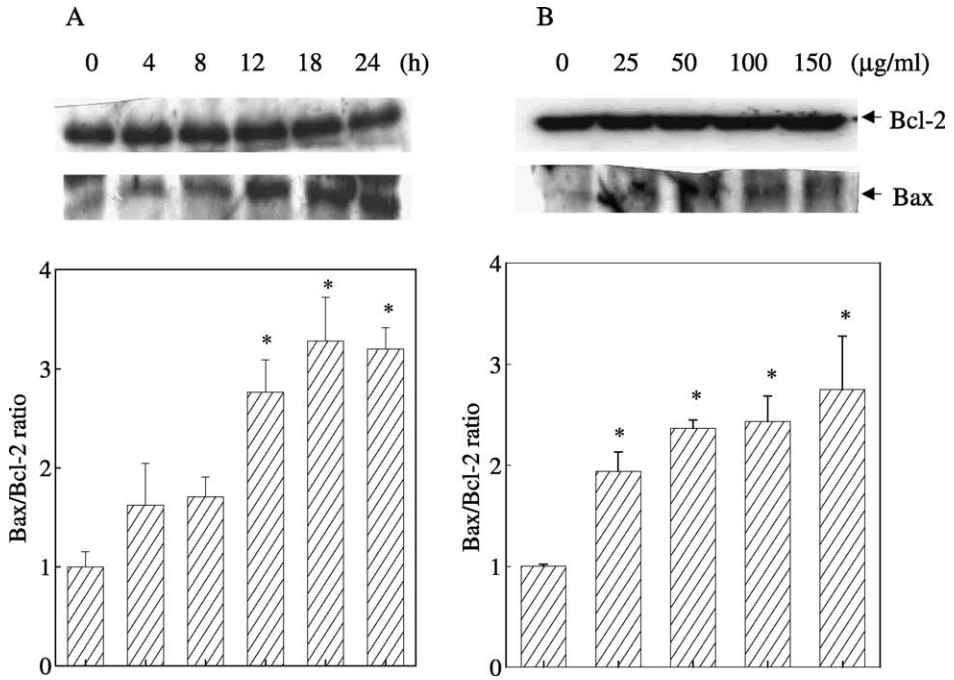


Fig. 7. Western blot analysis of Bcl-2 and Bax protein levels after exposure to *A. camphorata*. (A) MCF-7 cells treated with 150 μg/ml of *A. camphorata* at 0, 4, 8, 12, 18, and 24 h. (B) MCF-7 cells treated with 0, 25, 50, 100, and 150 μg/ml of *A. camphorata* for 24 h. Protein (50 μg) from each sample was resolved on 10% SDS-PAGE, and western blot performed. Typical result from three independent experiments is shown. Relative changes in Bcl-2 and Bax protein bands were measured using densitometric analysis. * indicates significant difference in comparison to control group ($P < 0.05$).

also play an important role in apoptosis by regulating the activity of certain enzymes involved in the cell-death pathway [44–48]. All these factors point to a significant role for intracellular oxidative metabolites in the regulation of apoptosis. Growth inhibition and ROS generation induced by *A. camphorata* in MCF-7 cells indicates that ROS production was probably the cause of this apoptic cell death.

The results of our previous study suggested that *A. camphorata* might possess protective antioxidant properties [14]. However, as described above, *A. camphorata* induced ROS generation in the MCF-7 cellular environment. Thus, the active components in *A. camphorata* might serve as a mediator of the reactive oxygen scavenging system and have the potential to act as a prooxidant and an antioxidant, depending on the redox state of the biological environment. Such a dual-property role for antioxidants has also been reported previously [52,53]. In addition, several researchers have shown that antioxidants, such as retinoids and vitamin E, produce

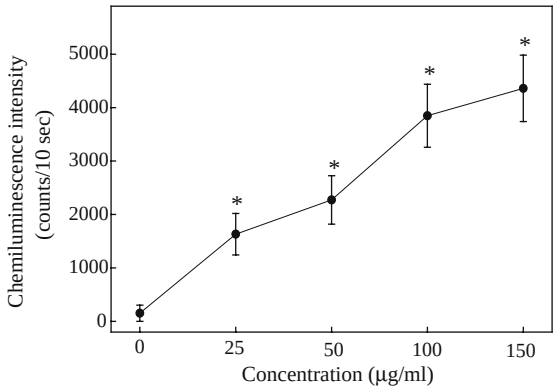


Fig. 8. ROS generation in *A. camphorata*-treated MCF-7 cells. MCF-7 cells were treated with 0, 25, 50, 100, and 150 μg/ml of *A. camphorata* for 24 h at 37 °C. Light emission was measured for a total period of 10 min under luminol as a chemiluminigenic probe using an ultrasensitive chemiluminescence analyzer. Values were calculated as total counts minus the background counts per 10 s. Results are mean ± SEM of three assays. * indicates significant difference in comparison to control group ($P < 0.05$).

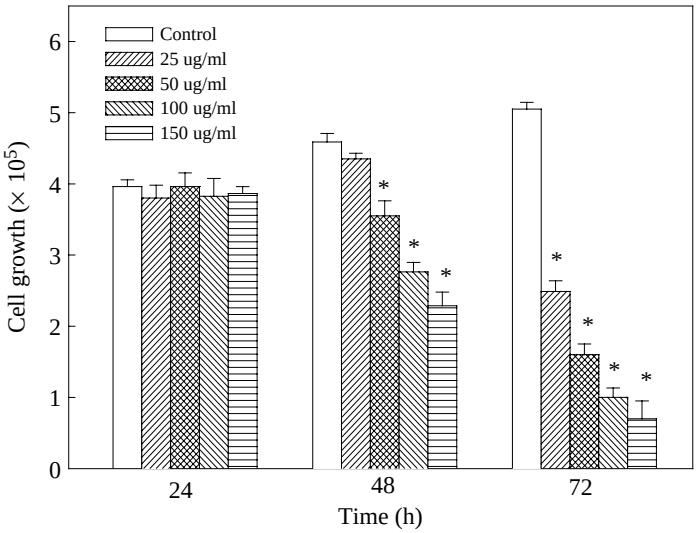


Fig. 9. Effects of *A. camphorata* on cell growth of HBL100 cells. Cells were incubated with 0, 25, 50, 100, and 150 μg/ml of *A. camphorata* for 24, 48, and 72 h. Cultures were then harvested, and cell numbers were obtained by counting cell suspensions with a hemocytometer. Results are presented as mean ± SEM of three assays. * indicates a significant difference in comparison to the control group ($P < 0.05$).

genetic changes that cause apoptosis in cancer cells by mechanisms other than antioxidant effect [54,55]. The detailed mechanisms of how *A. camphorata* acts on chemotherapy are unknown, and further investigations are needed.

Several active components, such as polysaccharides, triterpenoids, and, polyphenols have been isolated from mushrooms. Many studies have demonstrated that these active components, in both medicinal and edible mushrooms and plants, have various pharmacological properties, including antiinflammatory, antimutagenic, and anticarcinogenic activities [17–22]. Compounds isolated from *A. camphorata* include polysaccharides, ergostan-type triterpenoids, a sesquiterpene, and phenyl and biphenyl derivatives [56–58]. Song and Yen reported that the yields of polysaccharides, crude triterpenoids, and total polyphenols were approximately 23.2%, 47 and 67 mg/g, respectively [16]. In contrast, no polysaccharides, total polyphenols, or crude triterpenoids were detected in the dry matter of the culture medium. It seems reasonable to suggest, therefore, that *A. camphorata* metabolizes the culture medium and produces active components, such as polysaccharides, crude triterpenoids, and total polyphenols during the fermentation process of the submerged culture. These results imply that higher contents of polysaccharides, natural triterpenoids, and polyphenols the most-effective fraction of *A. camphorata* extracts, possibly act as chemopreventive agents with respect to inhibition of the growth of breast cancer cells through the induction of apoptosis. However, further investigation is required to identify the main active components of *A. camphorata*.

In conclusion, *A. camphorata* exhibits an antiproliferative effect by induction of apoptosis that is associated with cytochrome *c* translocation, caspase 3 activation, PARP degradation, and dysregulation of Bcl-2 and Bax in MCF-7 cells. As apoptosis has become a new therapeutic target in cancer research, these results confirm the potential of *A. camphorata* as an agent of chemotherapeutic and cytostatic activity in human breast cancer cells. However, further investigation of its activity, in vivo, is necessary to elaborate and exploit this nascent promise.

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