Food and Chemical Toxicology 46 (2008) 2680–2688

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Antrodia camphorata inhibits proliferation of human breast cancer cells *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 19 October 2007
Accepted 22 April 2008

Keywords:
Antrodia Camphorata
Breast cancer
Cell cycle arrest
Apoptosis

ABSTRACT

Antrodia camphorata (*A. camphorata*) has been shown to induce apoptosis in cultured human breast cancer cells (MDA-MB-231). In this study, we report the effectiveness of the fermented culture broth of *A. camphorata* in terms of tumor regression as determined using both *in vitro* cell culture and *in vivo* athymic nude mice models of breast cancer. We found that the *A. camphorata* treatment decreased the proliferation of MDA-MB-231 cells by arresting progression through the G1 phase of the cell cycle. This cell cycle blockade was associated with reductions in cyclin D1, cyclin E, CDK4, cyclin A, and proliferating cell nuclear antigen (PCNA), and increased CDK inhibitor p27/KIP and p21/WAF1 in a dose and time-dependent manner. Furthermore, the *A. camphorata* treatment was effective in delaying tumor incidence in the nude mice inoculated with MDA-MB-231 cells as well as reducing the tumor burden when compared to controls. *A. camphorata* treatment also inhibited proliferation (cyclin D1 and PCNA) and induced apoptosis (Bcl-2 and TUNEL) when the tumor tissue sections were examined histologically and immunohistochemically. These results suggest that the *A. camphorata* treatment induced cell cycle arrest and apoptosis of human breast cancer cells both *in vitro* and *in vivo*.

1. Introduction

A new basidiomycete, *Antrodia camphorata* (*A. camphorata*), in the Polyporaceae (Aphyllophorales), which causes brown heart rot in *Cinnamomum kanehirai* hay (Lauraceae) in Taiwan, has been identified as distinct species of the genus *Antrodia* (Zang and Su, 1990; Wu et al., 1997). *A. camphorata* (AC) is rare and expensive, as it grows only on the inner heart-wood wall of *Cinnamomum 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 cancer (Tsai and Liaw, 1985); however, very few biological activity tests are reported.

Chemoprevention, which refers to the administration of naturally occurring agents to prevent initiation and promotion events associated with carcinogenesis, is being increasingly appreciated as an effective approach for the management of neoplasia (Surh, 2003; Sporn and Suh, 2000). Many studies have shown associations between abnormal cell cycle regulation and apoptosis, and cancer, in as much as the cell cycle inhibitors and apoptosis-inducing agents are being appreciated as weapons for the management of cancer (Evan and Vousden, 2001; Stewart et al., 2003; Schmitt, 2003). Eukaryotic cell cycle progression involves the sequential activation of cyclin-dependent kinases (CDKs) whose activation is dependent upon their association with cyclins (Sherr, 1994). Progression through the mammalian mitotic cycle is controlled by multiple holoenzymes comprising a catalytic CDK and a cyclin regulatory subunit (Sherr, 1994). These cyclin-CDK complexes are activated at specific intervals during the cell cycle but can also be induced and regulated by exogenous factors. Cell cycle progression is also regulated by the relative balance between the cellular concentrations of CDK inhibitors, including P27/KIP and p21/WAF1 (Sandal et al., 2002). The cyclin-CDK complexes are subjected to inhibition via binding with CDK inhibitors (Sandal et al., 2002). 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 alteration of the normal apoptotic pathways (Bold et al., 1997). Apoptosis, a phenomenon associated with many physiological and pathological processes including cancer, has gained recognition as an ideal way to eliminate precancerous and cancer cells (Kiechle and Zhang, 2002; Nicholson, 2000). Thus, anticancer (chemopreventive) agents may alter regulation of the

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0278-6915/$ - see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.fct.2008.04.036

cell cycle machinery, resulting in an arrest of cells in different phases of the cell cycle and, thereby, reducing growth and proliferation of, and even inducing apoptosis in, cancerous cells, which may be useful in cancer therapy.

A. camphorata is well known in Taiwan as a traditional Chinese medicine, and it has been shown to exhibit antioxidant and anticancer effects. In our previous study, *A. camphorata* in submerged culture was used for the inhibition of AAPH-induced oxidative hemolysis and lipid/protein peroxidation of normal human erythrocytes (Hseu et al., 2002). Moreover, it has been demonstrated that *A. camphorata* protects low-density lipoproteins (LDL) against oxidative modification and may provide effective protection from atherosclerosis (Yang et al., 2006a). Our previous results also indicate that *A. camphorata* inhibits LPS induction of cytokines, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression by blocking NF-κB activation in RAW 264.7 macrophages (Hseu et al., 2005). Interestingly, a number of our studies have shown that *A. camphorata* exhibits significant apoptotic cell death in estrogen-nonresponsive MDA-MB-231 cells, estrogen-responsive MCF-7 cells, and premyelocytic leukemia (HL-60) cells (Hseu et al., 2004, 2007; Yang et al.,2006b). The *A. camphorata* concentration required for 50% inhibition of growth (IC50) was 136 and 316 μg/mL at 24 h for MDA-MB-231 and MCF-7 cells, respectively (Hseu et al., 2007). The effects were observed in breast cancer MDA-MB-231 and MCF-7 cells, and leukemia HL-60 cells, but not in healthy breast cells (HBL100), erythrocytes or human umbilical vein endothelial cells (Hseu et al., 2004; Yang et al., 2006b).

Breast cancer is the most common malignancy in American and northwestern European women. Approximately one-third of all females with breast cancer develops metastases and ultimately expires due to the effects of the disease. Recent progress in diagnosis and therapy has increased the survival rate of women with estrogen-dependent breast cancer. However, the treatment options available for estrogen-independent tumors are far from satisfactory, and consequently carry a poorer prognosis. This study investigates the anticancer effect of the fermented culture broth of *A. camphorata* harvested from submerged culture in terms of tumor regression using both *in vitro* cell culture (estrogen-independent, highly invasive, metastatic MDA-MB-231 cells) and *in vivo* athymic nude mice models of breast cancer. Additionally, the levels of cell cycle control and related molecules were assayed to establish the *A. camphorata* anticancer mechanism.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle's medium (GIBCO BRL, Grand Island, NY), antibody against Bcl-2, cyclin E, PCNA, CDC2 and p27/KIP (Santa Cruz Biotechnology Inc., Heidelberg, Germany), antibody against β-actin (Sigma Chemical Co., St. Louis, MO), and antibody against cyclin D1, CDK4, cyclin A and p21/WAF1 (Cell Signaling Technology Inc., Danvers, MA) were obtained from their respective suppliers. All other chemicals were of the highest grade commercially available and supplied either by Merck (Darmstadt, Germany) 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 a flask with 50-mL sterile water. The fragmented mycelia suspension was used as the inoculum after homogenization. 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 of mycelia inoculum was inoculated into a 250-L agitated fermentor (BioTop). The fermentation conditions were the same as those used for the seed fermentation, but at an aeration rate of 0.075 vvm. The fermentation product was then harvested at hour 331 and poured through a piece of non-woven fabric stretched over a 20-mesh sieve to separate the deep-red fermented culture broth and mycelia, and then centrifuged at 3000g 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 18.4 g/L. For preparation of the aqueous solution, the powder samples were solubilized with phosphate-buffered saline (PBS) at 25 °C. The stock solution was stored at −20 °C before analysis for apoptotic properties. Food Industry Research and Development Institute in Taiwan has standardized the preparation of different batches of *A. camphorata* using polyphenols, triterpenoids, polysaccharides, and biological activities, etc.

2.3. Cell culture

The estrogen-nonresponsive human breast cancer cell line, MDA-MB-231 was obtained from the American type Culture Collection (Manassas, VA). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 1% penicillin–streptomycin–neomycin in a humidified incubator (5% CO_2 in air at 37 °C). Cultures were harvested and monitored for cell number by counting cell suspensions with a hemocytometer using phase contrast microscopy.

2.4. Flow cytometric analysis

Cellular DNA content was determined by flow cytometric analysis of propidium iodide (PI)-labeled cells (Hseu et al., 2007). After plates of cells (1×10^6 cells/60-mm dish) were grown to semiconfluence, cell growth was arrested by washing plates with growth media supplemented with 1% FBS. Growth arrest was maintained for 24 h. The cell cycle synchronized cells were then washed with PBS and restimulated to enter the G1 phase together by addition of growth media containing *A. camphorata*, without FBS. After treatment, the cells were collected by trypsinization, and fixed in ice-cold 70% ethanol at −20 °C overnight. The cells were resuspended in PBS containing 1% Triton X-100, 0.5 mg/mL RNase, and 4 μg/mL PI at 37 °C for 30 min. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a single argon-ion laser (488 nm) was used for flow cytometric analysis. Forward and right-angle light scatter, which are correlated with the size of the cell and the cytoplasmic complexity, respectively, were 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. The cell cycle was determined and analyzed using ModFit software (Verity Software House, Topsham, ME). 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.

2.5. Preparation of cell extracts and immunoblot analysis

MDA-MB-231 cells (1.0×10^6 cells/60 mm dish) were detached, washed once in cold 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 using Bio-Rad protein assay reagent, with bovine serum albumin as the standard; protein extracts were reconstituted in sample buffer (0.062 M Tris–HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol), and the mixture was boiled for 5 min. Equal amounts (50 μg) of the denatured proteins were loaded into each lane, separated on a 10 or 12% 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 antibody for 2 h before being developed using SuperSignal ULTRA chemiluminescence substrate (Pierce, Rockford, IL). Band intensities were quantified by densitometry with absorbance of the mixture at 540 nm determined using an ELISA plate reader. Western blot analysis, with antibody against cyclin D1, cyclin E, CDK4, cyclin A, and PCNA, CDC2, p27/KIP and p21/WAF1 was performed as described previously (Yang et al., 2006b).

2.6. Animal experiments

Female athymic nude mice (BALB/*c-nu*), 5–7 weeks of age, were purchased from GlycoNex Inc. in Taiwan and were maintained in cage housing in a specifically designed pathogen-free isolation facility with a 12/12-h light/dark cycle; the mice were provided rodent chow and water *ad libitum*. All experiments were conducted in accordance with the guidelines of the China Medical University Animal Ethics Research Board.

2.7. Tumor cell inoculation

MB-MDA-231 cells (5×10^6 cells in 200 μL matrix gel) were injected subcutaneously on the right hind flank. These cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 1% penicillin–streptomycin–neomycin in a humidified incubator (5% CO_2 in air at 37 °C). Experiments were carried out using cells less than 20 passages. Tumor volume, as determined by weekly caliper measurements of tumor length, width and depth, were calculated using the formula: length × width^2 × 1/2 (Collins et al., 2003). The three groups received intraperitoneal injections of AC

(0.2 mL/mouse) dissolved in PBS buffer at a dose of 55 or 110 mg/kg, and NS-398 (COX-2 inhibitor used as positive control) at a dose of 5 mg/kg every two days while the control group received daily injections of vehicle only. Following 5 weeks of treatment, the mice were sacrificed. The tumors were removed and weighed before fixing in 4% paraformaldehyde, sectioning and staining with hematoxylin-eosin for light microscopic analysis. Part of the tumor tissue was immediately frozen and the rest was fixed in 10% neutral-buffered formalin and embedded in paraffin. To monitor drug toxicity, the body weight of each animal was measured weekly. In addition, a pathologist examined the mouse organs, including liver, lungs and kidneys.

2.8. Immunohistochemical detection of cyclin D1, PCNA, and Bcl-2

Cyclin D1, PCNA and Bcl-2 expression in tissue sections was analyzed by immunostaining using a monoclonal antibody (Santa Cruz Biotechnology Inc.). Deparaffinized sections were rehydrated and treated with 10 mM citrate buffer (pH 6.0) at 90 °C for 20 min. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide in methanol followed by two washes with PBS. The sections were then treated with blocking buffer (Dako Co., USA) for 30 min followed by washing in PBS. The sections were incubated with mouse anti-cyclin D1, anti-PCNA and anti-Bcl-2 antibodies overnight at 4 °C. The slides were incubated with biotinylated secondary antibody (Dako Co.) for 30 min at room temperature with pre- and post-washes with PBS. Sections were incubated with conjugated horseradish peroxidase streptavidin complex for 30 min followed by another incubation with 3,3′-diaminobenzidine (DAB) substrate for peroxidase (Dako Co.) for 5 min and a PBS wash. The sections were counterstained with hematoxylin. Finally, the slides were washed, dehydrated and mounted for microscopic examination and enumeration or immunoreactive cells (brown).

2.9. In situ apoptosis detection by TUNEL staining

Apoptotic cell death in deparaffinized tissue sections was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) with the Klenow DNA fragmentation detection kit (Calbiochem, San Diego, CA) (Gavrieli et al., 1992). Briefly, sections were permeabilized with 20 μg/mL protease K in TBS, and endogenous peroxidase was inactivated by 3% H_2O_2 in methanol. Apoptosis was detected by labeling the 3′-OH ends of the fragmented DNA with biotin-dNTP using klenow at 37 °C for 1.5 h. The slides were then incubated with streptavidin horseradish peroxidase conjugate, followed by incubation with 3,3′-diaminobenzidine and H_2O_2. Apoptotic cells were identified by the dark brown nuclei observed under a light microscope.

2.10. Statistics

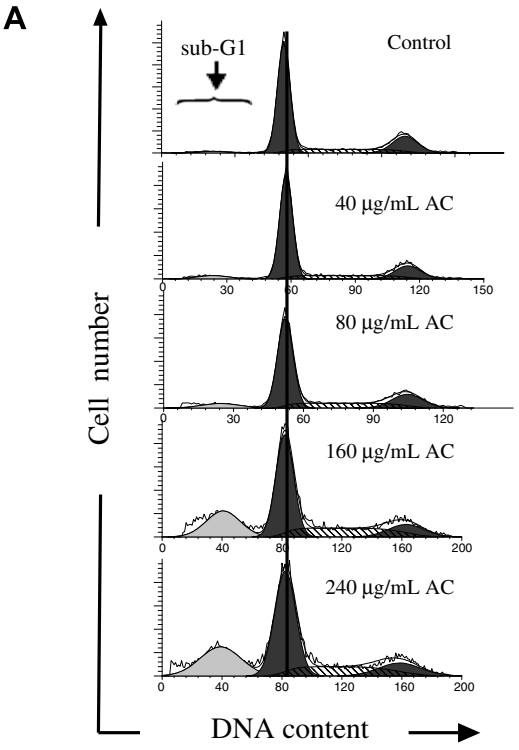
In vitro experiments are presented as mean and standard deviation (mean ± SD). For *in vivo* experiments, mean data values are presented with standard error (mean ± SE). All study data were analyzed using analysis of variance (ANOVA), followed by Dunnett's test for pairwise comparison. Statistical significance was defined as $p < 0.05$ for all tests.

3. Results

This study investigates the anticancer effect of the fermented culture broth of *A. camphorata* (harvested from submerged cultures) both *in vitro* and *in vivo* using the MDA-MB-231 breast carcinoma cell line and nude mice xenograft models.

3.1. Effect of A. camphorata on sub-G1 and cell cycle in MDA-MB-231 cells

The profile of the DNA content of the *A. camphorata*-treated MDA-MB-231 cells was obtained using flow cytometric analysis to measure the fluorescence of PI-DNA binding. MDA-MB-231 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 MDA-MB-231 cells (0–240 μg/mL for 24 h) when compared with the untreated group (Fig. 1A). Furthermore, the stage at which *A. camphorata*-induced growth inhibition occurs in the MDA-MB-231 cell cycle progression was determined, with cellular distribution in the different phases the treatment. Fig. 1B shows that *A. camphorata* exposure resulted in a progressive and sustained accumulation of cells in the G1 phase in a dose-dependent manner. Further, the percentage of G1 phase cells increased,



B

Apoptotic cells		Non-apoptotic cells		
μg/mL	(sub-G1)	G1	S	G2/M
0	4.1 ± 0.9	54.8 ± 1.3	34.8 ± 1.9	10.6 ± 1.5
40	7.0 ± 1.1	59.2 ±4.5	33.1 ± 1.1	7.0 ± 1.7 *
80	10.1 ± 2.1 *	68.0 ± 2.2 *	27.7 ± 2.7 *	4.6 ± 1.7 *
160	21.8 ± 3.2 *	67.6 ± 1.5 *	27.9 ± 2.4 *	5.1 ± 1.6 *
240	26.1 ± 2.9 *	69.2 ± 1.3 *	26.9 ± 1.1 *	3.9 ± 2.1 *

Fig. 1. Effects of *A. camphorata* on MDA-MB-231 cell cycle distribution. (A) Cells were treated with 0, 40, 80, 160 and 240 μg/mL of *A. camphorata* for 24 h, stained with PI, and analyzed for sub-G1 and cell cycle using flow cytometry. Representative flow cytometry patterns are shown. (B) Cellular distribution (as percentage) in different phases of the cell cycle (sub-G1, G1, S and G2/M) after *A. camphorata* treatment is shown. Apoptotic nuclei were identified as a subploid DNA peak and distinguished from cell debris on the basis of forward light scatter and PI fluorescence. Results are presented as meanSD of three assays. * Indicates significant difference in comparison to control group ($p < 0.05$).

while those in the S and G2/M phases decreased after treatment with *A. camphorata* (Fig. 1B). Our findings suggest that *A. camphorata* promotes cell growth inhibition by inducing G1 phase arrest in cancer cells.

3.2. Effect of A. camphorata on the expression of cyclin D1, cyclin E, CDK 4, cyclin A, and PCNA

In order to examine the molecular mechanism(s) and underlying changes in cell cycle patterns, we investigated the effects of various cyclins and CDKs involved in cell cycle control of MDA-MB-231 cells. We approached this study by treating MDA-MB-231 cells with *A. camphorata* (0–240 μg/mL) for 0–24 h. Dose and time-dependent reduction in cyclin D1, cyclin E, CDK4, and cyclin A expression with *A. camphorata* treatment were observed (Fig. 2A and B). Further, reduction in the expression of proliferating cell

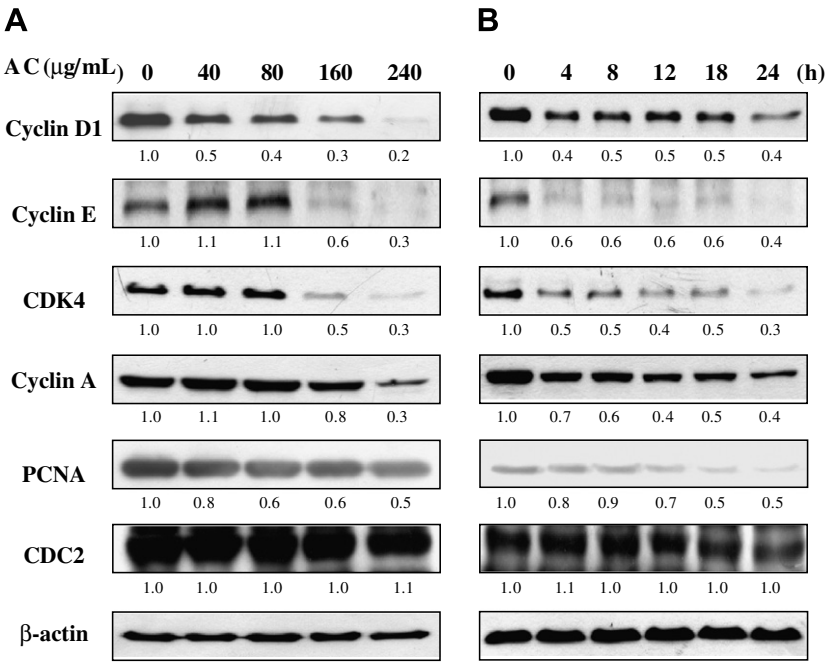


Fig. 2. Western blot analysis of cyclin D1, cyclin E, CDK4, cyclin A, PCNA, and CDC2 protein levels in MDA-MB-231 cells after exposure to *A. camphorata*. (A) Cells treated with 0, 40, 80, 160 and 240 μg/mL of *A. camphorata* for 24 h. (B) Cells treated with 160 μg/mL of *A. camphorata* for 0, 4, 8, 12, 18 and 24 h. Protein (50 μg) from each sample was resolved on 10% SDS-PAGE and western blot was performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical result from three independent experiments is shown.

nuclear antigen (PCNA), another marker of cell proliferation upregulated during the S phase, was also observed (Fig. 2A and B). Moreover, the experimental treatment did not appear to change the amount of detectable CDC2 protein (Fig. 2A and B). This result implies that *A. camphorata* inhibits cell cycle progression (G1 phase arrest) by reducing cyclin D1, cyclin E, CDK4, cyclin A, and PCNA.

3.3. A. camphorata increases P27/KIP and p21/WAF1 expression

The Cip/Kip family, including p27/KIP and p21/WAF1, binds to cyclin-CDK complexes and prevents kinase activation, subsequently blocking cell cycle progression (Sherr, 1995). As our investigation in the present study showed that *A. camphorata* treatment of breast cancer cells results in cell cycle arrest (G1 phase), we also examined the effect of *A. camphorata* exposure on cell cycle-regulatory molecules, including CDK inhibitor P27/KIP and p21/WAF1. As shown in Fig. 3A and B, treatment of MDA-MB-231 cells with *A. camphorata* (0–240 μg/mL for 0–24 h) induced marked dose and time-independent up-regulation of p27/KIP and p21/WAF1 protein.

3.4. Effect of A. camphorata on the growth of MDA-MB-231 xenograft in nude mice

Nude mice were used to evaluate the *in vivo* effect of *A. camphorata* on tumor growth. MDA-MB-231 cells were xenografted into nude mice as described in Section 2. All of the animals appeared healthy with no loss of body weight noted during *A. camphorata* treatment (Fig. 4A). In addition, no signs of toxicity were observed (body weight and microscopic examination of individual organs; data not shown) in any of the nude mice. The time course for MDA-MB-231 xenograft growth with *A. camphorata* (55/110 mg/kg) or without treatment (control) is shown in Fig. 4B. NS-398 (5 mg/kg COX-2 inhibitor) was used as a positive control. Evaluation of tumor volume showed significant dose and time-dependent growth inhibition associated with *A. camphorata* treatment (Fig. 4B). At the end of 5 weeks, the MDA-MB-231 xenograft tumor was excised from each sacrificed animal and weighed. Tumor weight in the *A. camphorata*-treated (55 or 110 mg/kg) mice was inhibited as compared with the control group (Fig. 5A and B). Additionally, microscopic examination of the tumor sections was used

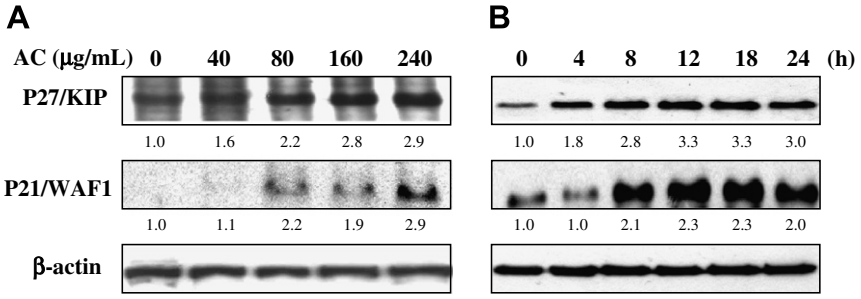


Fig. 3. Western blot analysis of P27/KIP and p21/WAF1 protein levels after exposure to *A. camphorata* in MDA-MB-231 cells. (A) Cells treated with 0, 40, 80, 160 and 240 μg/mL of *A. camphorata* for 24 h. (B) Cells treated with 160 μg/mL of *A. camphorata* for 0, 4, 8, 12, 18 and 24 h. Protein (50 μg) from each sample was resolved on 12% SDS-PAGE and western blot performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis. Typical result from three independent experiments is shown.

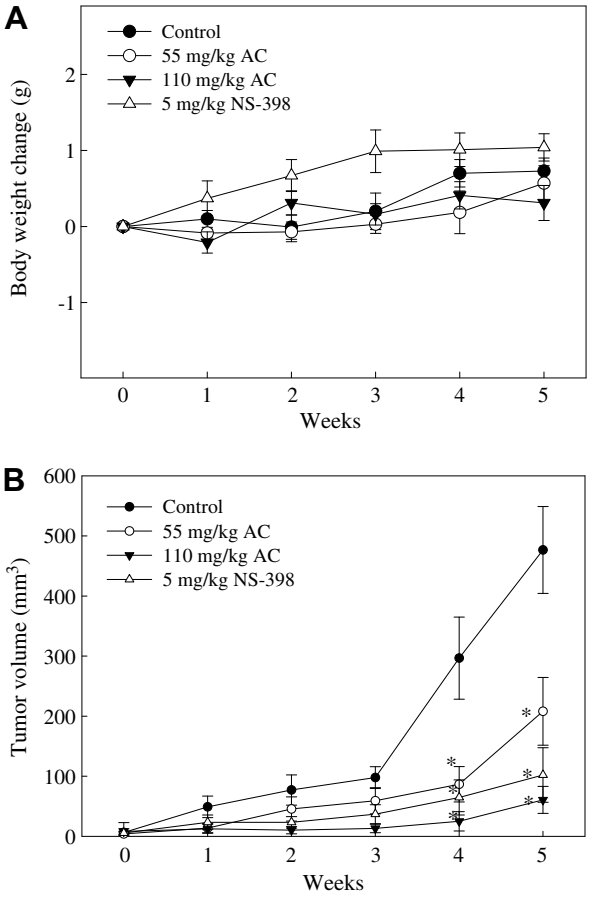


Fig. 4. Time-course effect of *A. camphorata* on growth of MDA-MB-231 xenograft was evaluated from weekly measurements of body weight change (A) and tumor volume (B). MDA-MB-231 cells were implanted subcutaneously into the flanks of nude mice on week 0, the animals were then treated with *A. camphorata* (55/110 mg/kg) or without (as control) as described in Section 2. NS-398 (COX-2 inhibitor, 5 mg/kg) was used as a positive control. Results are presented as meanSE ($n = 7$). * Indicates significant difference in comparison to control group ($p < 0.05$).

to distinguish differences in nucleic and cytoplasmic morphology after the 5 weeks of *A. camphorata* treatment. Furthermore, abundant mitosis was observed in the proliferating cells in the control group, while the number of mitosis-positive cells decreased in sections from the treated animals (data not shown). From the Fig. 5A, it can be noticed that the dose of 55 mg/kg is efficient in some but not all of the mice. This may be due to individual variation in the mice. These results demonstrate *A. camphorata*-related antitumor activity in nude mice bearing MDA-MB-231 breast cancer xenografts.

3.5. *A. camphorata* inhibits cell proliferation in xenograft tumors by immuno-histochemistry

As *A. camphorata* treatment was associated with a significant reduction in tumor volume, we further examined the effect on tumor growth by measuring cyclin D1 and PCNA, the markers of cell proliferation. Tumor sections were stained with anti-cyclin D1 and anti-PCNA, and the number of cyclin D1 and PCNA-positive cells in the mice xenografts was determined by histology and immunohistochemistry. Fig. 6A and B show that *A. camphorata* treatment significantly reduced the number of cyclin D1 and PCNA-positive cells in tumors from the *A. camphorata*-treated animals as compared to the controls, indicating the antiproliferative effect (Fig. 6A and B).

3.6. *A. camphorata* increases apoptosis in xenograft tumors

The effect of *A. camphorata* on tumor growth (apoptosis) in the MDA-MB-231 xenograft mice was also examined using Bcl-2 staining and TUNEL assay of the tumor sections. It has been shown that the Bcl-2 family plays an important regulatory role in apoptosis (Adams and Cory, 1998). Fig. 7A shows decreases in the number of Bcl-2-positive cells in tumors from *A. camphorata*-treated animals as compared to those from controls. The result indicates that *A. camphorata* may reduce Bcl-2 and, thus, lead to apoptosis in xenograft tumors. Furthermore, Fig. 7B shows that there were more TUNEL-positive cells in tumors from *A. camphorata*-treated animals relative to those from the untreated controls. These results demonstrate that *A. camphorata* treatment was associated with decreased proliferation and increased apoptosis in the study animals. Analysis of our data suggests that the *A. camphorata* promoted antitumor activity in nude mice bearing MDA-MB-231 breast cancer xenografts.

4. Discussion

Anticancer agents may alter regulation of the cell cycle machinery, resulting in cellular arrest at different phases of the cell cycle and, thereby, reducing the growth and proliferation of, and even inducing apoptosis in, cancerous cells. Several studies have demonstrated anticancer potential for extracts from a number of herbal medicines or mixtures *in vitro* or *in vivo* (Hu et al., 2002). Herbal medicine is one of the most ancient forms of health care known to humankind and it has been used in many cultures throughout history. Typically, herbal medicines emphasize the use of whole extracts from a plant mix or from complex formulations (Sporn and Suh, 2002). The present research documents a parallel study showing the effect of *A. camphorata* treatment *in vivo* in a human tumor xenograft in nude mice as well as in *in vitro* cell culture models involving estrogen receptor-negative MDA-MB-231. The data presented suggest that *A. camphorata* treatment may be effective in suppressing the proliferation of MDA-MB-231, a highly invasive estrogen receptor-negative breast cancer cell line, as shown by growth inhibition and apoptosis induction both *in vivo* and *in vitro*.

Investigation has shown the nontoxic characteristics of *A. camphorata* [oral administration of *A. camphorata* (500 mg/kg/day) for 28 days in rats], which increases its potential for application in food and drug products (Lin et al., 2001). Furthermore, *in vivo* toxicity was also examined superficially from body weight changes and histological study of vital organs (data not shown). There appeared to be no signs of significant toxicity at *A. camphorata* exposures of 55 and 110 mg/kg every two days. This likely indicates that there are no side effects at these doses. However, the tumour volume significantly had decreased already with a dose of 55 mg/kg whereas no effects were seen on the tumour weight, labelling indexes and anti-apoptotic index in nude animal studies. Tumor volume was determined by caliper measurements of tumor length, width and depth (Collins et al., 2003). We suggest that the differences between tumour volume and weight/labelling indexes/anti-apoptotic index could have occurred because the tumor had an anomalous shape. Because a dose of 110 mg/kg was more effective than a 55 mg/kg dose in the MDA-MB-231 xenograft model, we may adopt treatment with 110 mg/kg of *A. camphorata* to ensure drug compliance. However, future studies should test whether there is an optimal/effective dose between 110 and 55 mg/kg.

It has been demonstrated that differential regulation of the cell cycle and subsequent events leading to cell death account for the anticancer effect of some potential phytochemicals (Pozo-Guisado

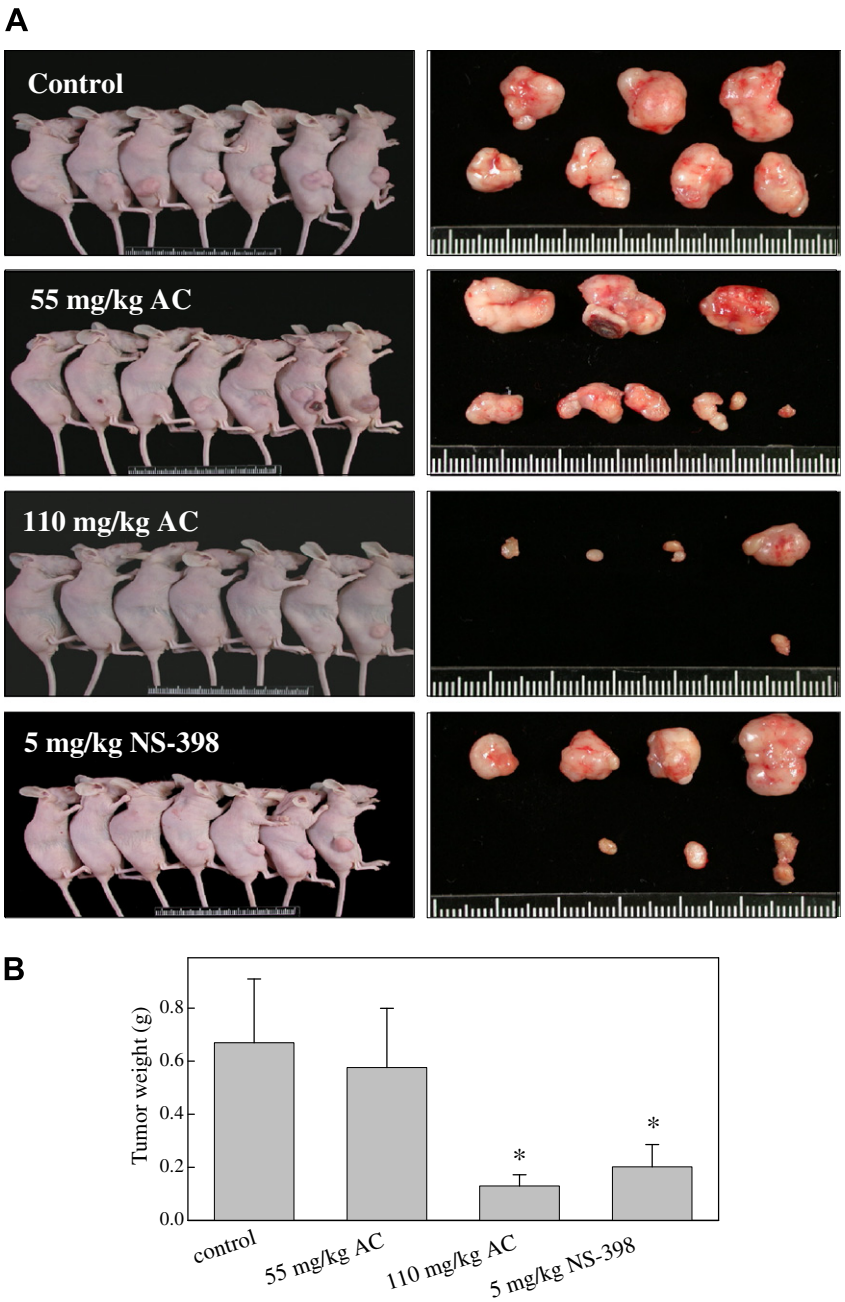


Fig. 5. *In vivo* inhibition of MDA-MB-231 xenograft proliferation by *A. camphorata*. Nude mice were treated with *A. camphorata* (55/110 mg/kg) or without (as control). NS-398 (COX-2 inhibitor, 5 mg/kg) was used as a positive control. On the fifth week after tumor implantation, the animals were sacrificed and the tumors were removed (A) and weighed (B). Results are presented as meanSE ($n = 7$). * Indicates significant difference in comparison to control group ($p < 0.05$).

et al., 2002; Jackson and Singletary, 2004). Disturbance of the cancer cell cycle is one of the therapeutic targets for development of new anticancer drugs (Carnero, 2002). The results of cell cycle analysis in the present study, as evaluated by flow cytometry, show that the *A. camphorata* treatment had a profound effect on cell cycle control, with the breast cancer cells accumulating in the G1 phase. Progression through the first gap phase (G1) requires both cyclin D-dependent CDK4/CDK6 and CDK2/cyclin E holoenzymes (Sherr, 1994). Furthermore, the A-type cyclins bind to and activate CDK2, are believed to be crucial to the regulation of S-phase entry, and appear to define the restriction point in the G1 phase (Elledge et al., 1992; Rosenblatt et al., 1992). Overexpression of cyclin D in the mammary gland of transgenic mice induces mammary carcinoma (Wang et al., 1994). Cyclin D expression is frequently deregulated in human neoplasias, and agents that can downregulate cyclin D expression could be helpful in both the prevention and treatment of human neoplasias (Sausville et al., 2000). Further, cyclin E, which is one of the key cell cycle regulators, has been found to be over-expressed in primary breast carcinoma tissue. Thus, it has potential as an independent predictor of the risk of visceral breast cancer relapse after surgery (Kim et al., 2001). Moreover, this cell cycle arrest has been associated with a decrease in the proliferating cell nuclear antigen (PCNA), an essential nuclear protein present in proliferating cells, used as a cell proliferation marker (Thangapazham et al., 2007; Paunesku et al., 2001). The results imply that the expression of D1, cyclin E, CDK4, cyclin A, and PCNA are down regulated in *A. camphorata*-treated MDA-MB-231 cells, which corroborates the G1 block.

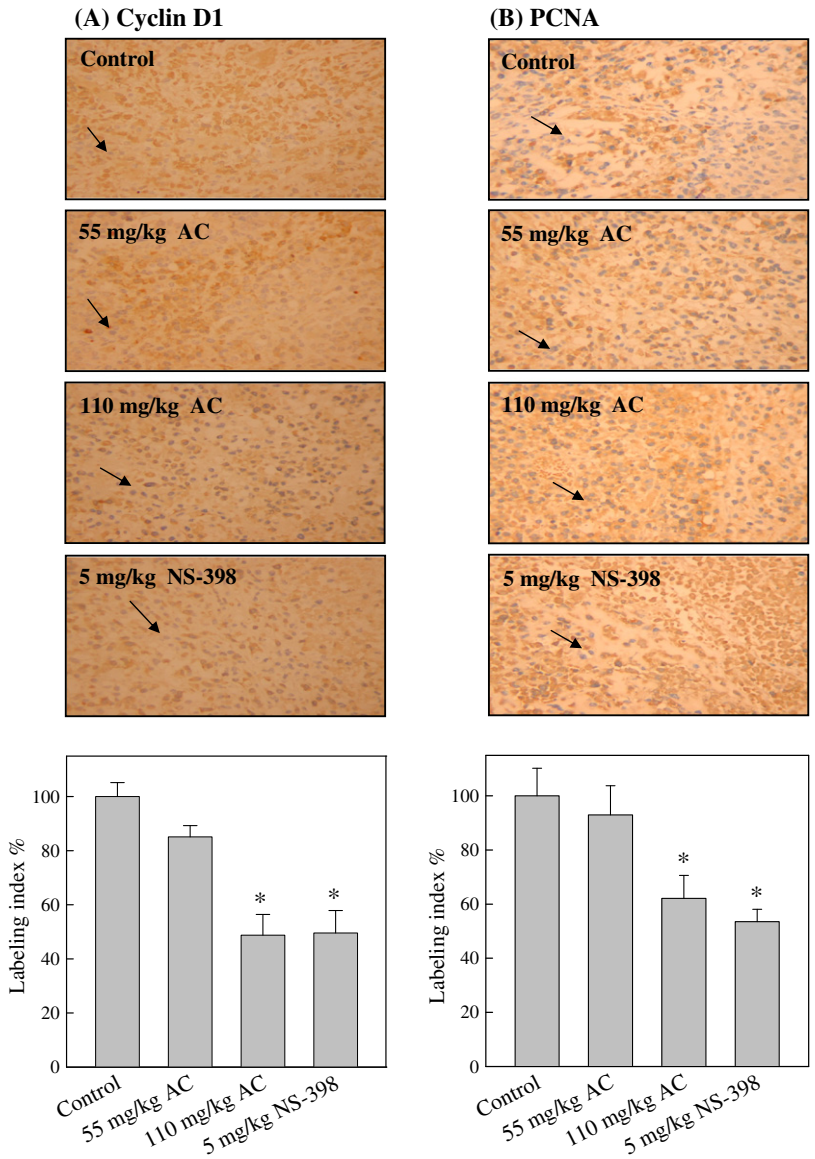


Fig. 6. Immunohistochemical analysis of proliferation in MDA-MB-231 xenograft tumors. Cyclin D1 (A) and PCNA (B) staining in tumor sections from control animals, and analogs treated with *A. camphorata* (55/110 mg/kg) or NS-398 (COX-2 inhibitor, 5 mg/kg). The proliferating cells were positively stained with monoclonal antibody against cyclin D1 and PCNA then counterstained with hematoxylin, as described in Section 2. Cells positive for cyclin D1 and PCNA were counted from 3 fields (200× magnification) for each tumor sample. The number of positive cells (arrows indicate proliferating cells) in microscopic fields from 5 to 7 samples was averaged. Results are the mean (±SE) number of cells/microscope field (as percentage) for 5–7 animals per group. * Indicates significant difference in comparison to control group ($p < 0.05$).

It has been shown that impairment of a growth stimulation signaling pathway induces the expression of CDK inhibitor which binds to and subsequently inhibits cyclin-CDK activity (Sandal et al., 2002). The CDK inhibitors, P27/KIP and p21/WAF1, regulate the activity of CDKs, which in turn regulate the phosphorylation of pRb and generally inhibit cell cycle progression (Sherr, 1995; Nigg, 1993). These results suggest that induction of P27/KIP and p21/WAF1 expression might account for a large part of the reduction in CDK activity and, subsequently, block cell cycle progression. Our study has also demonstrated that there were no significant differences in the expression of CDC2 after the *A. camphorata* treatment. The evidence suggests that the complex formed by the association of CDC2 and cyclin B1 plays a major role at entry into mitosis (Guadagno and Newport, 1996). Analysis of our data suggests that the observed inhibition of proliferation in MDA-MB-231cells associated with the *A. camphorata* treatment could be the result of cell cycle arrest during the G1 phase.

Apoptosis is an important homeostatic mechanism that balances cell division and cell death and maintains the appropriate number of cells in the body. Disturbances of apoptosis in cancer cells have been studied in detail, and induction of apoptosis in these cells is one of the strategies for anticancer drug development (Hu and Kavanagh, 2003). Our previous investigation has demonstrated that treatment of MDA-MB-231 cells with *A. camphorata* can induce the apoptotic cell death associated with internucleosomal DNA fragmentation, cytochrome c translocation, caspase-3, −8, and −9 activation, poly ADP-ribose polymerase (PARP) degradation, and dysregulation of Bcl-2 and Bax (Hseu et al., 2007). Furthermore, tumor inhibition by *A. camphorata* was also observed in the nude mice xenograft model in this study. Both incidence and mean tumor volume were significantly reduced by *A. camphorata* treatment. Immunohistochemical staining revealed decreased proliferation (cyclin D and PCNA) and increased apoptosis (Bcl-2 and TUNEL assay) in tumors from the *A. camphorata*-treated animals.

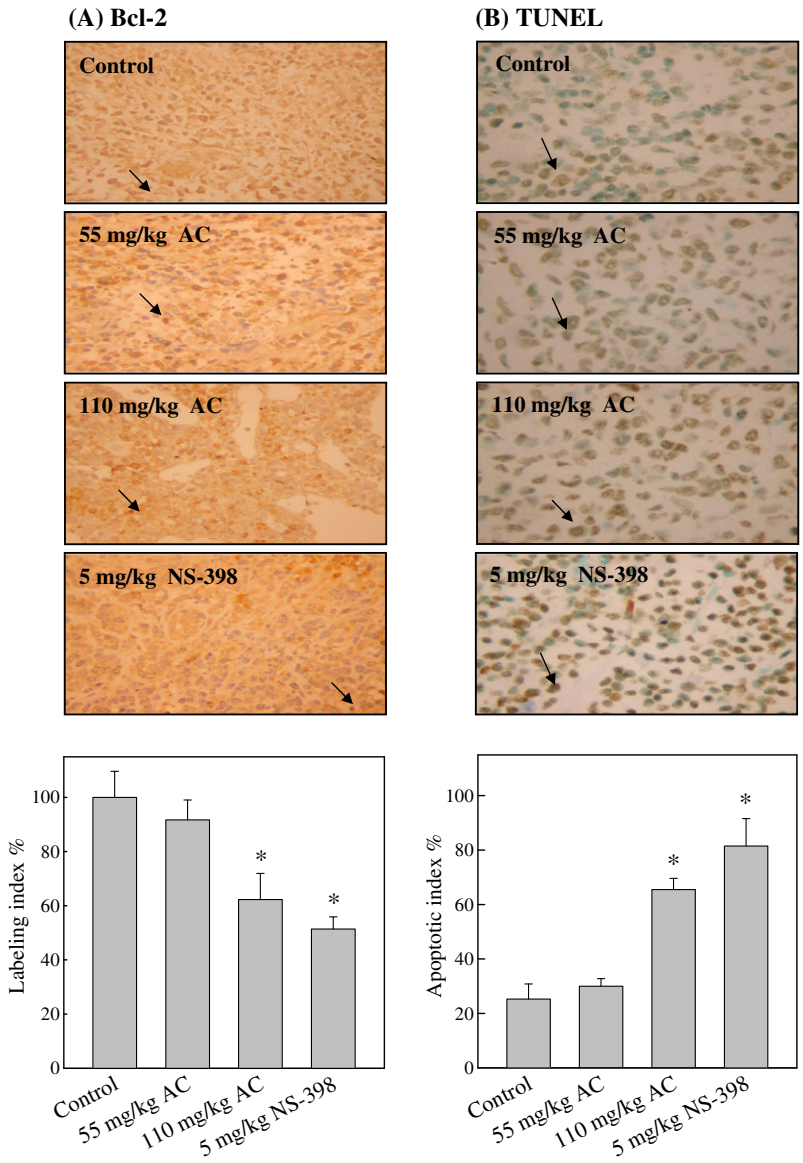


Fig. 7. Immunohistochemical staining of apoptosis (Bcl-2 and TUNEL) in MDA-MB-231 xenograft tumors. Bcl-2 staining (A) and *in situ* apoptosis detection using TUNEL staining (B) in tumor sections from control animals and experimental analogs treated with *A. camphorata* (55/110 mg/kg) or NS-398 (COX-2 inhibitor, 5 mg/kg). Cells positive for Bcl-2 (200×) and TUNEL (400×) were counted from 3 fields for each tumor sample. The number of Bcl-2 and TUNEL positive cells (arrows indicate apoptotic cells) in microscopic fields from 5 to 7 samples was averaged. Results are the mean (±SE) number of cells/microscope field (as percentage) for 5–7 animals per group. * Indicates significant difference in comparison to control group ($p < 0.05$).

Analysis of our study data suggests that *A. camphorata* induces cell cycle arrest and apoptosis induction in human breast cancer cells both *in vitro* and *in vivo*.

Natural products, including plants and microorganisms, provide rich resources for anticancer drug discovery (Schwartsmann et al., 2002). As the different components in a given herb may have synergistic activities or buffer toxic effects, mixtures or extracts of these herbs may offer greater therapeutic or preventive activity in combination (Vickers, 2002; Li et al., 2000). Scientific interest in the active compounds (polysaccharides, triterpenoids and polyphenols) isolated from mushrooms has recently been aroused due to their anti-inflammatory, antimutagenic, and anticarcinogenic properties. Compounds isolated from *A. camphorata* include polysaccharides, ergostan-type triterpenoids, a sesquiterpene, and phenyl and biphenyl derivatives (Chen and Yang, 1995). Reported yields of polysaccharides, crude triterpenoids and total polyphenols from the dry matter derived from the culture broth of *A. camphorata* were approximately 23.2%, and 47 and 67 mg/g, respectively (Song and Yen, 2002). In contrast, no polysaccharides, total polyphenols or crude triterpenoids were detected in the dry matter of the *A. camphorata* culture medium (Song and Yen, 2002). It was also determined that the cytotoxicity of the culture medium is lower than that of *A. camphorata* in submerged culture (Hseu et al., 2007), indicating that the active components must be derived from secondary metabolites of the mycelia. It seems reasonable to suggest, therefore, that *A. camphorata* metabolizes the culture medium and releases 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 fractions of *A. camphorata*, possibly act as potential chemopreventive agents with respect to inhibition of the proliferation (growth) of human breast cancer cells through the induction of both cell cycle arrest and apoptosis *in vitro* and *in*

vivo. Further investigation is required, however, to identify the main active constituents of *A. camphorata*.

These results suggest that *A. camphorata* may possess anticancer properties in human breast cancer. These data can be considered as an important step before going to patients or in food and drug products. However, further *in vivo* studies using animal models and human patients are necessary to elaborate and exploit this nascent promise.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants CMU96-112 and CMU96-207 from China Medical University, and NSC 96-2628-B-039-030-NY3 from the National Science Council of ROC.

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