



Anti-metastatic activities of *Antrrodia camphorata* against human breast cancer cells mediated through suppression of the MAPK signaling pathway

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ABSTRACT

The fermented culture broth of *Antrrodia camphorata* (*A. camphorata*) has been shown to promote cell cycle arrest and apoptosis of human estrogen-nonresponsive MDA-MB-231 cells. Herein, we demonstrate that non-cytotoxic concentrations (20–80 μ g/mL) of *A. camphorata* markedly inhibited the invasion/migration of highly metastatic MDA-MB-231 cells as shown by an *in vitro* transwell and a wound-healing repair assay. The results of a gelatin zymography assay showed that *A. camphorata* suppressed the activity of matrix metalloproteinase (MMP)-9 and urokinase plasminogen activator (uPA). Western blot results demonstrated that treatment with *A. camphorata* decreased the expression of MMP-9, MMP-2, uPA, uPA receptor (uPAR) and vascular endothelial growth factor (VEGF); while the expression of the endogenous inhibitors of these proteins, i.e., tissue inhibitors of MMP (TIMP-1 and TIMP-2), and plasminogen activator inhibitor (PAI)-1, increased. Further investigation revealed that *A. camphorata* suppressed the phosphorylation of ERK1/2, p38, and JNK1/2. *A. camphorata* treatment also led to a dose-dependent inhibition on NF- κ B binding and activation. This is the first report confirming the anti-metastatic activity of this potentially beneficial mushroom against human breast cancer.

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1. Introduction

Breast cancer is the most common malignancy in American and northwestern European women. Metastasis is the primary cause of breast cancer mortality. The 5-year survival rate for women diagnosed with localized breast cancer is 98%, which contrasts dramatically with the 27% survival rate of women diagnosed with distant metastatic breast cancer (Ries et al., 2007). The process of metastasis consists of sequential and selective steps including proliferation, induction of angiogenesis, detachment from the primary site, motility, invasion into the connective tissue and circulation, aggregation and survival in the circulation, cell arrest

in distant capillary beds and extravasation into organ parenchyma (Fidler, 1991). The principal mechanisms involved in cancer mortality are migration and invasion, where primary cancer cells disseminate and grow at a distant site resulting in a secondary tumor. When cancer cells invade and migrate, a number of proteolytic enzymes contribute to the degradation of environmental barriers, such as the extracellular matrix and the basement membrane. Thus, degradation of the extracellular matrix and components of the basement membrane, mediated by the concerted action of proteinases, such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), play a critical role in tumor invasion and metastasis (Westermarck and Kahari, 1999). Therefore, the inhibition of MMP- and/or uPA-mediated migration or invasion could be a potential treatment for preventing or inhibiting cancer metastasis.

Antrrodia camphorata (*A. camphorata*) is well known in Taiwan as a physiologically beneficial mushroom. It is a newly discovered basidiomycete of the family Polyporaceae (Aphyllphorales)

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that causes brown heart rot in the Taiwan native tree *Cinnamomum kanehirai* Hay (*Lauraceae*) and has been identified as a distinct species of the genus *Anrodia* (Zang and Su, 1990). It has been used in traditional Chinese medicine for the treatment of food poisoning and drug intoxication, diarrhea, abdominal pain, hypertension, skin itches, and liver cancer (Tsai and Liaw, 1985). There is increasing evidence that *A. camphorata* possesses an extensive range of biological activity, including anti-cancer, antioxidant, hepatoprotective, anti-hypertensive, anti-hyperlipidemic, immunomodulatory, and anti-inflammatory activities (Ao et al., 2009; Hseu et al., 2002, 2004, 2005, 2007, 2008a,b, 2010; Yang et al., 2006a,b; Chan et al., 2010; Hsieh et al., 2010). In our earlier studies, we found that the fermented broth of *A. camphorata*, harvested from submerged cultures, can induce significant apoptosis and cell cycle dysregulation in highly metastatic, estrogen-nonresponsive MDA-MB-231 cells *in vitro* and *in vivo* (Hseu et al., 2007, 2008a). The effects were observed in MDA-MB-231 cells, but not in healthy breast cells (HBL100) (Yang et al., 2006b). However, little is currently known about its effect against tumor invasion and metastasis. In this study, the ability of non-cytotoxic concentrations (20–80 µg/mL) of *A. camphorata* to inhibit metastasis of human breast cancer MDA-MB-231 cells was investigated. Additionally, the biochemical steps linking *A. camphorata* to the metastatic process in these cells was also investigated.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine and penicillin/streptomycin/neomycin were obtained from GIBCO BRL (Grand Island, NY). Antibodies against MMP-2, MMP-9, uPA, uPAR, TIMP-1, TIMP-2, and PAI-1 were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). The β -actin monoclonal antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The polyclonal antibody to TIMP-2 was purchased from R&D Systems (Minneapolis, MN, USA). Phospho-extracellular signal-regulated kinase 1/2 (pERK1/2), ERK1/2, phospho-p38 (pp38), p38, phospho-c-Jun N-terminal kinase (pJNK1/2), and JNK1/2 antibodies were purchased from Cell Signaling (Beverly, MA, USA). All other chemicals were of the highest grade commercially available, and were supplied either by Merck (Darmstadt, Germany) or Sigma.

2.2. Preparation of the fermented culture broth of *A. camphorata* from submerged cultures

The *A. camphorata* culture was inoculated on potato dextrose agar and incubated at 30 °C for 15–20 days. The whole colony was then put into a flask containing 50 mL sterile water. After homogenization, the fragmented mycelial suspension was used as an inoculum. The seed culture was prepared in a 20 L fermentor (BioTop) agitated at 150 rpm with an aeration rate of 0.2 vvm at 30 °C. A 5-day culture of 15 L of mycelium inoculum was inoculated into a 250 L agitated fermentor (BioTop). The fermentation conditions were the same as those used for the seed fermentation, but the aeration rate was 0.075 vvm. The fermentation product was harvested at hour 331 and poured through a non-woven fabric on a 20-mesh sieve to separate the deep-red fermented culture broth and the 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 a powder. The yield of dry matter from the culture broth was 18.4 g/L. For preparation of the stock solution, the powder samples were solubilized with DMEM containing 1% FBS (pH 7.4). The stock solution (1.6 mg/mL) was stored at –20 °C before evaluation of its anti-metastatic properties. The experiments were done using 2–4 different batches of the fermented culture of *A. camphorata* (Hseu et al., 2010). The HPLC profile of the fermented culture broth of *A. camphorata* was performed as previously described (Hseu et al., 2010). We refer to the fermented culture broth of *A. camphorata* as *A. camphorata* throughout the manuscript.

2.3. Cell culture

The MDA-MB-231 breast cancer cell line, which is an estrogen-independent, highly metastatic human breast cancer cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were grown in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 1%

penicillin–streptomycin–neomycin at 37 °C in a humidified incubator with 5% CO₂. Cultures were harvested and monitored for changes in cell number by counting cell suspensions using a hemocytometer with phase contrast microscopy.

2.4. MTT assay

Cell viability was monitored by the MTT colorimetric assay. Briefly, cells (2.5×10^5 cells/24-well dish) were treated with up to 160 µg/mL *A. camphorata* for 24 h before 400 µL 0.5 mg/mL MTT in PBS was added to each well. After incubation at 37 °C for 4 h, an equal cell culture volume of 10% SDS (400 µL) was added to dissolve the MTT formazan, and the absorbance was measured at 570 nm (A_{570}). Cell viability (%) was calculated as:

$$(A_{570} \text{ of treated cells} / A_{570} \text{ of untreated cells}) \times 100$$

2.5. Cell invasion assay

Invasion assays were performed using BD Matrigel invasion chambers (Bedford, MA, USA). For the invasion assay, 10 µL Matrigel (25 mg/50 mL) was applied to an 8-µm polycarbonate membrane filters, and the bottom chamber of the apparatus contained standard medium. Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth–Holm–Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. Briefly, the top chambers were seeded with 1×10^5 cells in 500 µL serum-free medium, and the cells were incubated with up to 80 µg/mL *A. camphorata*. The bottom chambers (750 µL) were filled with media supplemented with 10% FBS. Cells were allowed to migrate for 24 h at 37 °C. After the incubation, the non-migrated cells on the top surface of the membrane were removed with a cotton swab. The migrated cells on the bottom side of the membrane were fixed in cold 75% methanol for 15 min and washed three times with PBS. The cells were stained with Giemsa stain solution and then de-stained with PBS. Images were obtained using an optical microscope (200 × magnification), and invading cells were quantified by manual counting. Percentage inhibition of invading cells was quantified, with untreated (control) cells representing 100%. To determine the importance of TIMP-2 in *A. camphorata*-mediated anti-invasive effects, TIMP-2 activity was blocked with a neutralizing antibody. The MDA-MB-231 cells were treated with 80 µg/mL *A. camphorata* for 24 h in the presence or absence of a 2.5 µg/mL anti-TIMP-2 neutralizing antibody (R&D Systems); cultures were pretreated with the neutralizing antibody for 30 min in a Matrigel invasion assay.

2.6. *In vitro* wound-healing repair assay

For the cell migration assay, 3×10^5 MDA-MB-231 cells were seeded into a 12-well culture dish and grown in DMEM containing 10% FBS to a nearly confluent cell monolayer. The cells were resuspended in DMEM medium containing 1% FBS. The monolayers were carefully scratched using a 200 µL pipette tip. Cellular debris was removed by washing with PBS, and then the cells were incubated with up to 80 µg/mL *A. camphorata* for 24 h. The migrated cells were fixed in cold 75% methanol for 30 min and washed three times with PBS. The cells were then stained with Giemsa stain solution and de-stained with PBS. The cultures were photographed (100 × magnification) at 0 and 24 h to monitor the migration of cells into the wounded area, and the closure of wounded area was calculated.

2.7. Gelatin zymography assay

The activities of MMP-2, MMP-9, and uPA in the medium released from MDA-MB-231 cells were measured by gelatin zymography protease assays. MDA-MB-231 cells (3×10^5 cells) were seeded into 12-well culture dishes and grown in DMEM containing 10% FBS to a nearly confluent cell monolayer. The cells were resuspended in DMEM containing 1% FBS, and then incubated with up to 80 µg/mL *A. camphorata* for 24 h. Briefly, collected media of an appropriate volume (adjusted by vital cell number) were prepared with SDS sample buffer without boiling or reduction, and 1 mg/mL gelatin or casein for MMPs or for uPA, respectively, was added and subjected to 8% SDS–PAGE electrophoresis. After electrophoresis, gels were washed with 2.5% Triton X-100 and then incubated in the developing buffer (50 mM Tris-base, 200 mM NaCl, 5 mM CaCl₂ and 0.02% Brij 35) at 37 °C for 24 h. Then, the gels were stained with Coomassie brilliant blue R-250.

2.8. Western blot analysis

One million MDA-MB-231 cells per 60 mm dish were grown in DMEM containing 10% FBS to a nearly confluent monolayer, and resuspended in DMEM containing 1% FBS. The cells were incubated with up to 80 µg/mL *A. camphorata* for 24 h, detached and washed once in cold PBS, and then resuspended in 100 µL lysis buffer (10 mM Tris–HCl [pH 8], 0.32 M sucrose, 1% Triton X-100, 5 mM EDTA, 2 mM dithiothreitol, and 1 mM phenylmethyl sulfonyl fluoride). The suspension was put on ice for 20 min, and then centrifuged at 16,000g for 20 min at 4 °C. Total protein content was determined using the 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% sodium dodecylsulfate (SDS), 10% glycerol, and 5% β -mercaptoethanol), and the mixture was boiled for 5 min. Equal amounts (50 μ g) of the denatured proteins were loaded into each lane, separated on 8–15% SDS polyacrylamide gels, followed by transfer of the proteins to polyvinylidene difluoride (PVDF) membranes overnight. Membranes were blocked with 0.1% Tween-20 in PBS containing 5% non-fat dried 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 enzyme-linked immunosorbent assay (ELISA) plate reader. Western blot analyses, with antibodies against MMP-9, MMP-2, uPA, uPAR, TIMP-1, TIMP-2, PAI-1, VEGF, pERK1/2, ERK1/2, pp38, pJNK1/2, or JNK1/2 were performed as described previously (Hseu et al., 2008a). Protein lysates (30 μ g) were loaded onto 12% polyacrylamide gels to determine the expression of ERK1/2, p38, and JNK1/2 mitogen-activated protein kinases. Proteins were electrophoresed and electrotransferred as described previously (Simeone et al., 2004).

2.9. NF- κ B binding assay

Binding of NF- κ B in the nuclear extracts was assessed using an electrophoretic mobility shift assay (EMSA) with biotin-labeled double-stranded NF- κ B oligonucleotides using an EMSA kit (Panomics, CA, USA). MDA-MB-231 cells (3×10^5 cells) were seeded into 12-well culture dishes and grown in DMEM containing 10% FBS. The cells were resuspended in DMEM containing 1% FBS, and then incubated with up to 80 μ g/mL *A. camphorata* for 24 h. Binding reactions containing 10 μ g of nuclear protein, 10 mM Tris, 50 mM KCl, 1 mM DTT, 5 mM MgCl₂, 1 mg poly (dI-dC) and 2 pmol oligonucleotide probe were incubated for 30 min at 15 °C. Specific binding was confirmed by using a 200-fold excess of unlabeled probe as a specific competitor. Protein-DNA complexes were separated by using a 6% non-denaturing acrylamide gel electrophoresis at 4 °C, and then transferred to positively charged nylon membranes (Millipore, MA, USA) and fixed by baking the membrane for one hour in an oven at 80 °C. Gel shifts were visualized with a streptavidin-horseradish peroxidase followed by chemiluminescent detection.

2.10. NF- κ B activation assay

MDA-MB-231 cells were grown in DMEM medium containing 10% FBS, 3×10^5 cells/12-well dish. After an overnight incubation, pNF- κ B-SEAP was cotransfected with the pRES-hrGFP-1a expression vector (10:1) into cells using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA). After an 8 h post-transfection, the cells were resuspended in serum-free medium, and then incubated with up to 80 μ g/mL *A. camphorata* for 24 h. SEAP activity in the medium was evaluated by using the Phospha-Light™ System according to the protocol of the manufacture (Applied Biosystems, Bedford, MA, USA).

2.11. Statistical analyses

Results are presented as mean \pm standard deviation (mean \pm SD). All study data were analyzed using analysis of variance, followed by Dunnett's test for pair-wise comparison. Statistical significance was defined as $p < .05$ for all tests.

3. Results

In this study, MDA-MB-231 human breast cancer cells were used to investigate the ability of *A. camphorata* to inhibit invasion and metastasis, and to elaborate the molecular mechanisms involved.

3.1. *A. camphorata* reduces the viability of MDA-MB-231 cells

In our previous study, we found that *A. camphorata* promoted growth inhibition of MDA-MB-231 cells. The *A. camphorata* concentration required for 50% inhibition of growth (IC₅₀) by *A. camphorata* was 136 μ g/mL after a 24 h incubation for MDA-MB-231 cells (Hseu et al., 2007). To investigate the effects of a low dose of *A. camphorata* on the viability of MDA-MB-231 cells, the cells were exposed to up to 160 μ g/mL *A. camphorata* for 24 h, and evaluated using an MTT assay (Fig. 1). At 24 h, concentrations of 20–80 μ g/mL did not affect the number of cells; however, 100–160 μ g/mL proved to be cytotoxic ($p < .05$), as previously reported. These observations confirm and extend the anti-proliferating activity of *A. camphorata* on MDA-MB-231 cells. The results also

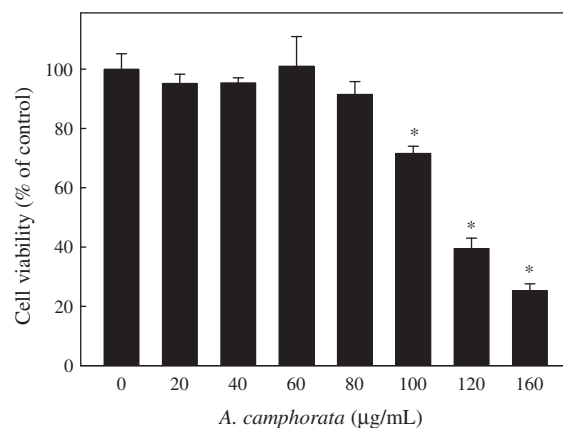


Fig. 1. *A. camphorata* reduces the viability of MDA-MB-231 cells. Cells were either untreated or treated with 20, 40, 60, 80, 100, 120, 140, or 160 μ g/mL of *A. camphorata* for 24 h. Results are presented as mean \pm SD of three assays. *Significant difference in comparison to the control group ($p < .05$).

suggested that non-cytotoxic concentrations (20–80 μ g/mL) of *A. camphorata* could be used to evaluate its anti-metastatic properties.

3.2. *A. camphorata* inhibits in vitro invasion and migration of MDA-MB-231 cells

Matrigel has been used by numerous groups to assay the invasive activity of tumor cells across the basement membrane. Therefore, a transwell assay was used to investigate the invasion potential of MDA-MB-231 cells 24 h after treatment with 20–80 μ g/mL *A. camphorata*, which significantly decreased ($p < .05$) the invasion potential of MDA-MB-231 cells in a dose-dependent manner (Fig. 2A). Furthermore, to determine the effects of *A. camphorata* on in vitro MDA-MB-231 cell migration, confluent monolayers of MDA-MB-231 cells were scraped to remove a section of monolayer and cultured for 24 h with control buffer plus *A. camphorata* (up to 80 μ g/mL). The results indicated that *A. camphorata* significantly decreased the migration of MDA-MB-231 cells in a dose-dependent manner (Fig. 2B) ($p < .05$).

3.3. *A. camphorata* reduces MMP-9 and uPA activity

MMPs and uPA, which are involved in the degradation of the basement membrane, are essential to the invasive process. To examine the effect of *A. camphorata* on MMPs and uPA levels, MDA-MB-231 cells were treated with up to 80 μ g/mL *A. camphorata* for 24 h. The supernatant was collected and examined for MMP-2, MMP-9, and uPA activity using gelatin zymography assays. As shown in Fig. 3A and B, treatment with *A. camphorata* decreased the MMP-9 and uPA activity in MDA-MB-231 cells in a dose-dependent manner ($p < .05$). However, the experimental treatment did not appear to change the amount of detectable MMP-2 protein.

3.4. *A. camphorata* mediates the downregulation of MMP-9, MMP-2, uPA, uPAR, and VEGF expression

Using Western blotting, we analyzed the effects of treating cells with up to 80 μ g/mL *A. camphorata* for 24 h on MMPs, uPA, and uPAR protein expression. Treatment of MDA-MB-231 cells with *A. camphorata* induced noticeable reductions in MMP-9, MMP-2, uPA, and uPAR protein expression (Fig. 4A). Furthermore, western blot analysis of VEGF expression showed that VEGF expression was downregulated following treatment with *A. camphorata* (Fig. 4C).

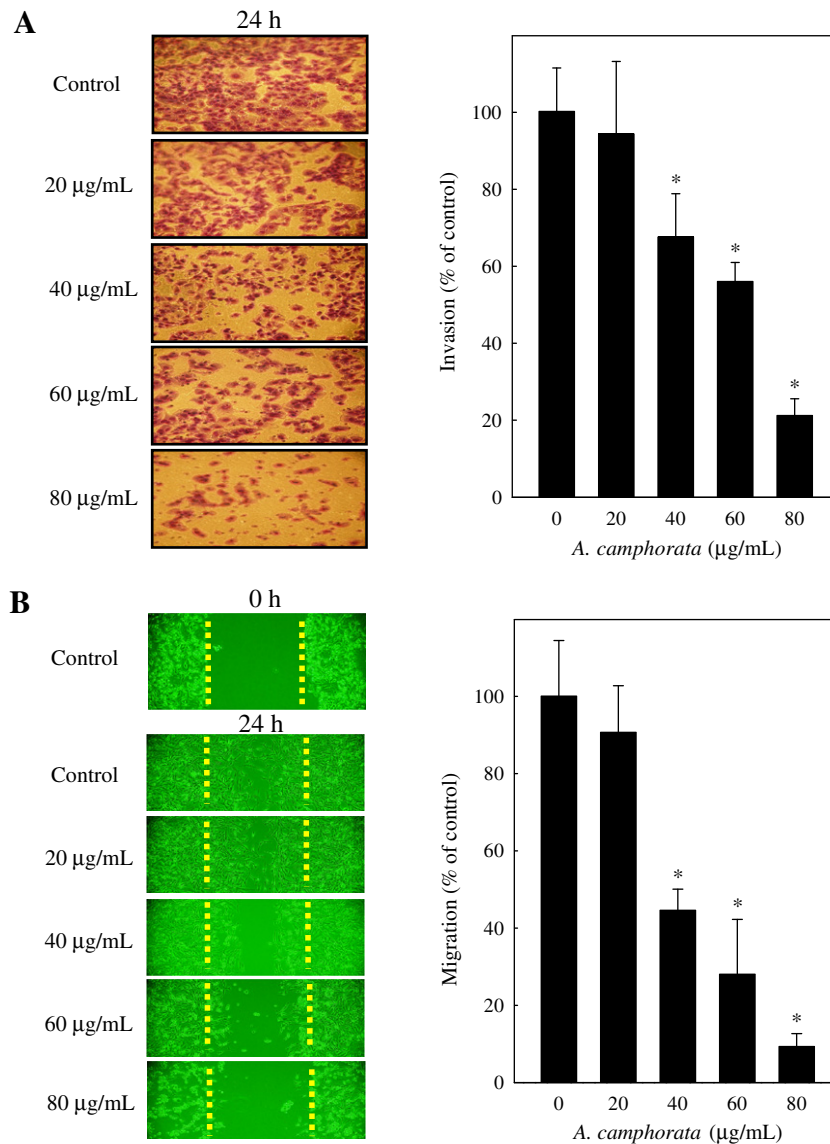


Fig. 2. *A. camphorata* inhibits the invasion and migration of MDA-MB-231 cells in an *in vitro* transwell assay and a wound-healing repair assay as described in Section 2. (A) MDA-MB-231 cells were pretreated with up to 80 µg/mL *A. camphorata*, and, after 24 h, cells invading under the membrane were photographed (200 × magnification). The percentage inhibition of invading cells was quantified and expressed on the basis that untreated cells (control) represented 100%. Invasiveness was determined by counting cells in three microscopic fields per sample. (B) Cells were scratched and treated with up to 80 µg/mL *A. camphorata*, and migration was observed using a phase-contrast microscope (100 × magnification) at 0 and 24 h, and the closure of area was calculated. Results are presented as mean ± SD of three assays. *Significant difference in comparison to control group ($p < .05$).

3.5. *A. camphorata* increases protein levels of TIMP-1, TIMP-2, and PAI-1

Physiological activity of MMPs and uPA is greatly related to that of TIMPs and PAIs, respectively, their specific endogenous inhibitors. Therefore, we investigated the effect of treatment of MDA-MB-231 cells with up to 80 µg/mL *A. camphorata* for 24 h on the expression of TIMPs and PAIs. A marked upregulation of TIMP-1, TIMP-2, and PAI-1 expression was observed following treatment with *A. camphorata* (Fig. 4B).

3.6. *A. camphorata* increases TIMP-2 production to block breast cancer cells from invading through Matrigel

Next, we determined the importance of TIMP-2 in the anti-invasive effects of *A. camphorata*. To do this, TIMP-2 activity was

blocked with a commercially available neutralizing antibody and the effect of exposure to up to 80 µg/mL *A. camphorata* for 24 h on the invasiveness of MDA-MB-231 cells in Matrigel was determined. At the concentration used, the anti-TIMP-2 antibody had no effect on invasion (Fig. 5A and B). *A. camphorata* decreased the invasiveness of MDA-MB-231 cells in Matrigel; however, blocking TIMP-2 activity significantly ($p < .05$) suppressed the anti-invasive effects of *A. camphorata* (Fig. 5A and B). Compared to untreated MDA-MB-231 cells, *A. camphorata*-treated cells displayed a decrease in number of invasive cells by 16%; introduction of the neutralizing anti-TIMP-2 antibody reduced this to 51% (Fig. 5B). These data indicated that TIMP-2 is an important mediator of the anti-invasive activity of *A. camphorata* against the MDA-MB-231 cells in the Matrigel basement membrane. Notably, one mechanism by which *A. camphorata* inhibits breast cancer cell invasion is the upregulation of TIMP-2 production.

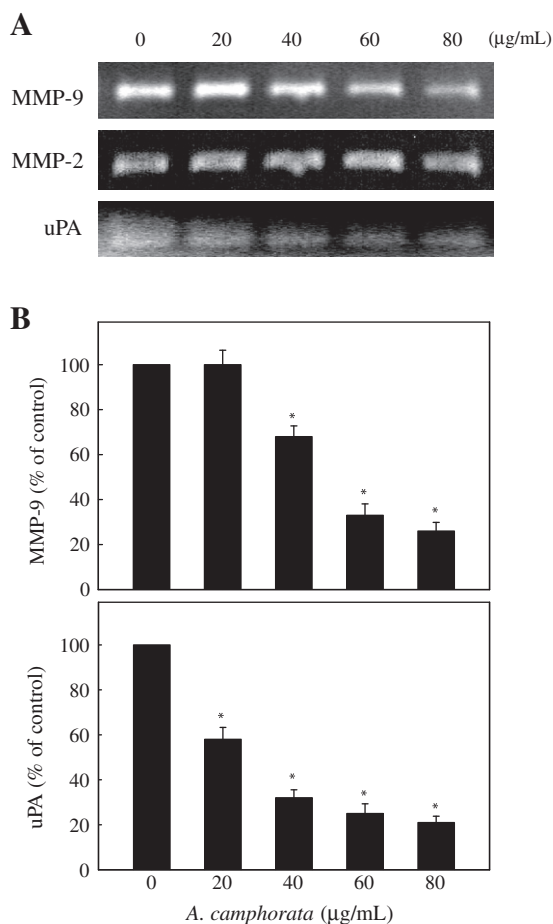


Fig. 3. Inhibitory effects of *A. camphorata* on the activities of MMP-9 and uPA of MDA-MB-231 cells. (A) Cells were treated with up to 80 µg/mL *A. camphorata* for 24 h and then subjected to gelatin zymography to analyze the activities of MMP-9, MMP-2, and uPA. (B) The activities of these proteins were subsequently quantified by densitometric analysis. Typical results from three independent experiments are shown. *Significant difference in comparison to control group ($p < .05$).

3.7. *A. camphorata* suppresses MAPK signaling pathway

We next sought to determine if *A. camphorata* treatment affects intracellular signal transduction that is essential for stimulating cell invasion and migration, the MAPK signaling pathway. We found that treating MDA-MB-231 cells with up to 80 µg/mL *A. camphorata* for 24 h suppressed activation of ERK1/2, p38, and JNK1/2 (Fig. 6A and B) ($p < .05$). This suggested that *A. camphorata* might inhibit the invasion and migration of MDA-MB-231 cells through the suppression of the MAPK signaling pathway.

3.8. An inhibitory effect of *A. camphorata* on NF-κB DNA binding and activation

NF-κB family of transcription factors are known to regulate numerous genes involved in MMP, uPA, or VEGF secretion, as well as the downstream effectors of the MAPK signaling pathway (Chen et al., 2001; Kim et al., 2006). To examine the effect of *A. camphorata* on the activation of NF-κB, MDA-MB-231 cells were treated with 20–80 µg/mL *A. camphorata* for 24 h. NF-κB DNA binding activity was determined using nuclear extracts and EMSA; *A. camphorata* treatment dose-dependently inhibited the DNA binding activity of NF-κB (Fig. 7A). Based on these results, it is possible that *A. camphorata* treatment inhibits NF-κB binding to its DNA re-

sponse element. To investigate if *A. camphorata* affected the transcriptional regulation of NF-κB, MDA-MB-231 cells were transfected with pNF-κB-SEAP containing three tandem NF-κB binding sites upstream of the SEAP gene and then treated with 20–80 µg/mL *A. camphorata* for 24 h. SEAP activity was measured using a commercially available chemiluminescence assay. The result of a SEAP reporter assay showed that the SEAP activity decreased markedly after treatment with *A. camphorata* (Fig. 7B) ($p < .05$). Analysis of our data indicated that binding activities of NF-κB were strongly decreased by *A. camphorata*.

4. Discussion

Approximately one-third of all women with breast cancer develops metastasis and ultimately dies as a result of the effects of the disease. Thus, metastasis has been a major challenge for the successful treatment of this cancer. This study revealed that *A. camphorata* could significantly inhibit the migration ability of MDA-MB-231 cancer cells and demonstrated the inhibitory effect of *A. camphorata* on breast cancer invasiveness. According to our literature search, this is the first scientific report examining the inhibitory effects of *A. camphorata* on (MDA-MB-231) breast cancer invasiveness, and these observations confirm and extend the anti-metastatic action of *A. camphorata*.

In this study, the levels of metastatic control and associated molecules were assayed to determine the *A. camphorata* anti-metastatic mechanism. MMPs and uPA, which are secreted by invasive cancer cells, play important roles in cancer cell invasion and metastasis because tumor cells must cross the type IV collagen-rich basement membrane of vessel walls to spread to other sites (Stacker et al., 2002). Further, physiological activities of MMPs and uPA are related closely to that of their specific endogenous inhibitors, TIMPs and PAIs, respectively (Stacker et al., 2002). Inhibition of invasion mediated by MMPs and uPA plays a key feature in the prevention of cancer metastasis. Tumor cell models of breast cancer have demonstrated that increasing MMP expression levels correlate with increasing aggressiveness of tumor cell growth and metastatic potential (Bachmeier et al., 2001). The control of MMP synthesis and activation can therefore be regarded as an important target for the prevention of tumor progression. Among the MMPs, MMP-9 and MMP-2, which are abundantly expressed in various malignant tumors, are considered to play critical roles in tumor invasion and metastasis (John and Tuszyński, 2001). Moreover, there is substantial evidence on the inhibition of MMP-9 and suppression of invasiveness and metastases of cancer cells using various chemopreventive or chemotherapeutic agents (Ho et al., 2002; Abiru et al., 2002). Our present findings demonstrate that *A. camphorata* could decrease the activity or protein levels of tumor metastasis-related proteins, including MMP-9, MMP-2, uPA, and uPAR, and increase the expression of their endogenous inhibitors, TIMP-1, TIMP-2, and PAI-1, in MDA-MB-231 cells. Therefore, *A. camphorata* could be a potential agent for the prevention of breast cancer metastasis.

Cell invasion involves MMP-mediated proteolysis of the basement membrane, which is counterbalanced by TIMPs. Thus, the possible usefulness of specifically selected MMP inhibitor, would be worthy of investigation also as chemopreventive agents in patients at high risk of developing cancer. Generally, activation of MMP-9 is primarily regulated by the balance between proenzyme activation and inhibition by TIMP-1 (Brown et al., 1990), whereas MMP-2 is constitutively expressed and secreted as a latent zymogen, pro-MMP-2. Its activation occurs on the cell membrane through the formation of a trimolecular complex composed of the membrane-type MMP (MT1-MMP), TIMP-2 and pro-MMP-2 (Egeblad and Werb, 2002). High levels of TIMP-2 have been

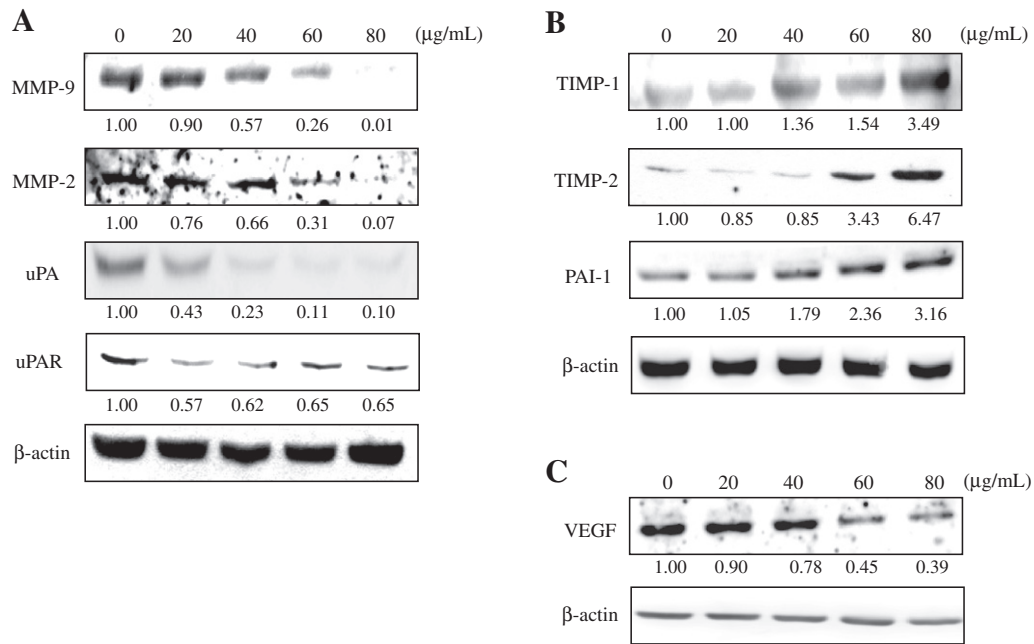


Fig. 4. *A. camphorata* mediates the downregulation of MMP-9, MMP-2, uPA, uPAR, and VEGF expression, and the upregulation of their endogenous inhibitors. Western blot analysis of the protein levels of MMP-9, MMP-2, uPA, and uPAR (A), and their endogenous inhibitors TIMP-1, TIMP-2, and PAI-1 (B) in MDA-MB-231 cells after exposure to *A. camphorata*. (C) *A. camphorata* reduced VEGF expression in MDA-MB-231 cells. Cells were treated with up to 80 μg/mL of *A. camphorata* for 24 h. Proteins (50 μg) from each sample were resolved on 8–15% SDS-PAGE, and Western blot was performed. β-actin was used as a control. Relative changes in protein bands were measured using densitometric analysis with the control being 100% as shown just below the gel data. Typical results from three independent experiments are shown.

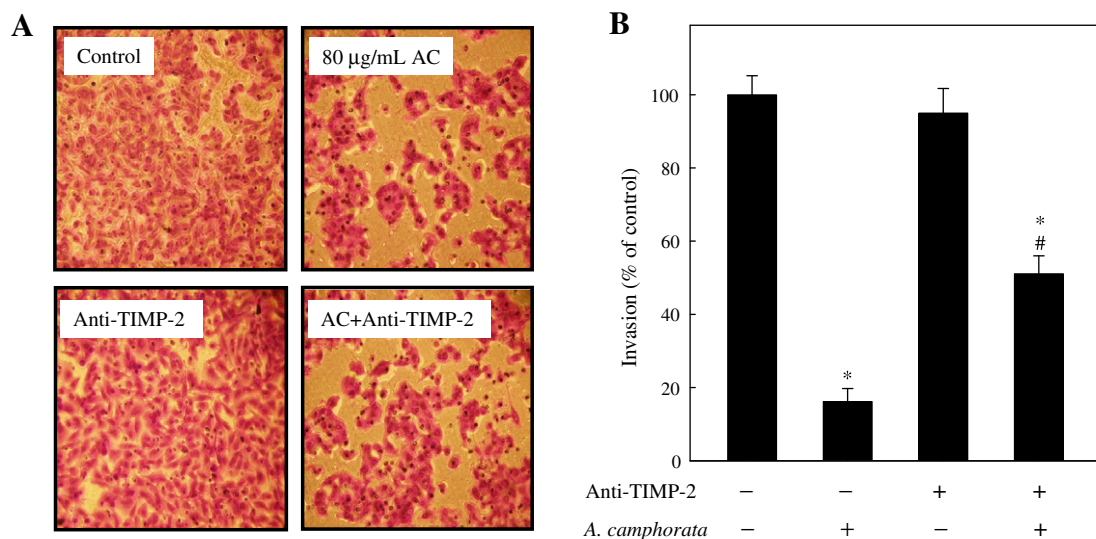


Fig. 5. *A. camphorata* increases TIMP-2 production to suppress breast cancer invasion through Matrigel by using a transwell assay as described in Section 2. MDA-MB-231 cells were treated with *A. camphorata* (80 μg/mL) for 24 h in the presence or absence of 1 μM TIMP-2 neutralizing antibody. Results are presented as mean ± SD of three assays. *#Significant difference in comparison to control and *A. camphorata* alone group group ($p < .05$). AC: *A. camphorata*.

correlated with distant metastasis of breast tumors (Vizoso et al., 2007). Overexpression of TIMP-2 decreased the *in vitro* invasion of Ras-transformed breast epithelial cells (Ahn et al., 2004). Mice injected with TIMP-2-transfected MDA-MB-231 breast cancer cells developed a lower number of osteolytic bone metastases and had a higher survival rate than mice injected with non-transfected cells (Yoneda et al., 1997). In the present study, we found that blocking TIMP-2 activity with a neutralizing antibody significantly increased the invasive activity of *A. camphorata*-treated MDA-MB-231 cells. Our findings are consistent with of the idea that TIMP-2 acts as a suppressor of cell invasion. Since inhibition of

TIMP-2 did not fully block the anti-invasive effects of *A. camphorata*, other mechanisms are likely to be involved in the anti-invasive effects of *A. camphorata*.

In addition, we also found that *A. camphorata* inhibited the expression of a primary angiogenic cytokine, VEGF, by MDA-MB-231 cells. Transformed epithelial cells have been shown to be the major source of VEGF expression in many types of solid cancers (Guidi et al., 1997; Abu-Jawdeh et al., 1996). Over-expression of VEGF is linked to increased angiogenesis and more aggressive tumor behavior (McLeskey et al., 1998), anti-angiogenic interventions based on VEGF antibodies or disruption of signal transduction

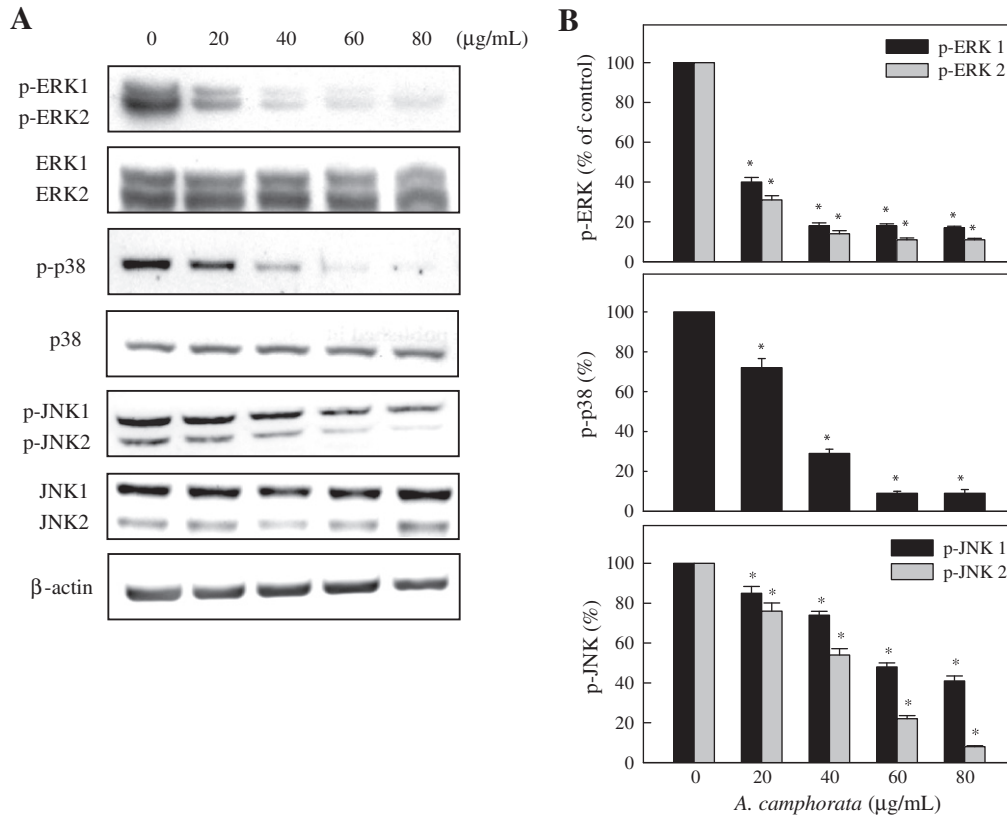


Fig. 6. The MAPK signaling pathway is suppressed by *A. camphorata* treatment. The phosphorylation of ERK1/2, p38 MAPK, and JNK1/2 was inhibited in MDA-MB-231 cells following treatment with up to 80 μg/mL of *A. camphorata* for 24 h. The levels of indicated proteins in the cell lysates were analyzed by using specific antibodies, and the amounts of β-actin were used as internal controls for sample loading. Results are presented as mean ± SD of three assays. *Significant difference in comparison to control group ($p < .05$).

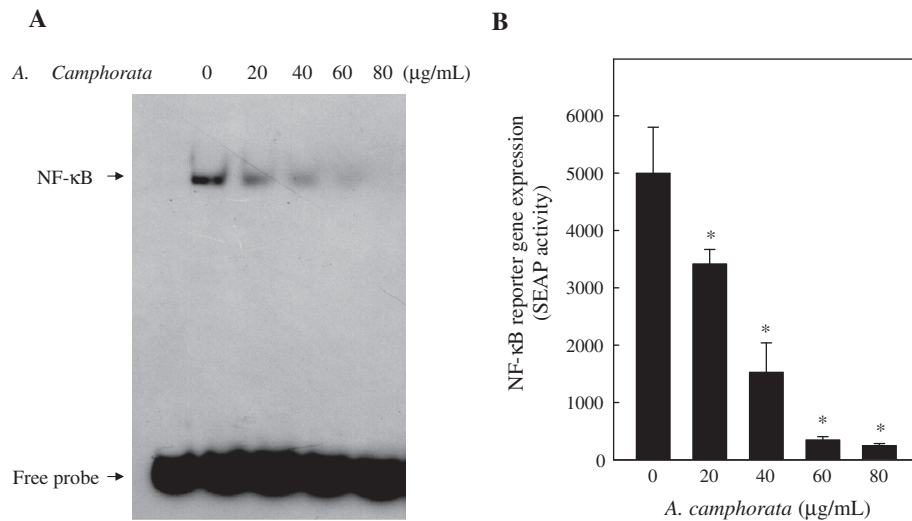


Fig. 7. *A. camphorata* inhibits the NF-κB DNA binding and activation. (A) MDA-MB-231 cells were treated with up to 80 μg/mL *A. camphorata* for 24 h, and then nuclear extracts were analyzed for NF-κB DNA binding activity using a biotin-labeled NF-κB-specific oligonucleotide for EMSA. Lane 1 represents nuclear extracts incubated with unlabeled oligonucleotide to confirm the specificity of binding. (B) The SEAP activity was measured after treatment of the pNF-κB-SEAP transfected MDA-MB-231 cells with up to 80 μg/mL *A. camphorata* for 24 h. Results are presented as mean ± SD of three assays. *Significant difference in comparison to control group ($p < .05$).

through its receptors have been shown to inhibit tumor growth (Benjamin et al., 1999). Accumulating evidence indicates that angiogenesis inhibitors are potential candidate drugs for cancer chemoprevention and therapy (Felmeden et al., 2003). The inhibitory effect of *A. camphorata* on VEGF expression in breast cancer cells,

therefore, may be an important mechanism in the regulation of neo-vascularization of human breast tumors, further contributing to the overall control of tumor growth and progression.

Since several studies using different cell types have indicated that MAPKs, like ERK, p38, and JNK, seem to play a central role in

regulating the expression of MMPs or uPA, inhibition of the MAPK pathway might also potentially prevent invasion and metastasis of a wide range of tumors (Westermarck and Kahari, 1999). Our present data demonstrates that *A. camphorata* treatment inhibited phosphorylation of ERK1/2, p38, or JNK1/2 and this resulted in a concurrent reduction in the levels of MMPs and uPA, indicating a possible mechanism of inhibition of MMPs or uPA synthesis by *A. camphorata*.

In the present study, we found that *A. camphorata* inhibited the DNA-binding and transcriptional activity of constitutively activated and inducible nuclear factor- κ B (NF- κ B). The activation of NF- κ B, which is downstream of the MAPK pathway, is involved in many pathological processes, such as inflammation, cancer cell adhesion, angiogenesis, invasion, and metastasis (Chen et al., 2001). Generally, NF- κ B is a key player in tumorigenesis, and inhibitors of NF- κ B activation have been shown to suppress MMPs, uPA, and VEGF, as well as further tumor invasion (Kim et al., 2006). Therefore, it has been suggested that suppression of the NF- κ B activities may allow for the blocking tumor initiation, promotion and metastasis, as well as the blocking of factors that bind to these regulatory elements; therefore, this represents an appropriate approach to inhibit the synthesis of MMPs, uPA or VEGF. Our findings suggest that *A. camphorata*-mediated inhibition of the invasiveness of MDA-MB-231 cells may be mediated, in part, through the suppression of MMPs, uPA, and VEGF expression via modulation of NF- κ B signaling pathway.

There is increasing evidence that the compounds identified in *A. camphorata* are predominantly polysaccharides, triterpenoids, steroids, benzenoids, and maleic/succinic acid derivatives (Ao et al., 2009; Geethangili and Tzeng, 2009). The reported yields of the fermented *A. camphorata* broth in terms of polysaccharides, crude triterpenoids, and total polyphenols are 23.2 mg/g, 47 mg/g, and 67 mg/g, respectively (Song and Yen, 2002). In contrast, no polysaccharides, crude triterpenoids, or total polyphenols are detected in the dry matter of the culture medium (Song and Yen, 2002). It has been demonstrated that ethylacetate extract from fruiting bodies of *A. camphorata* decreased the invasion of liver cancer (PLC/PRF/5) cells (Hsu et al., 2007). Moreover, both wound-scratch and transwell assays have demonstrated that the migration capability of T24 human bladder carcinoma cells is significantly retarded when exposed to *A. camphorata* extracts from fruiting bodies (Peng et al., 2006). It has been demonstrated that polysaccharides, triterpenoids, and polyphenols found in herbs, fruits, and vegetables could inhibit invasion and metastasis of cancer cells (Han et al., 1999). In the present study, we found that the fermented culture broth of *A. camphorata* from submerged cultures could significantly inhibit the metastatic ability of MDA-MB-231 cells. These observations confirm and expand the reported anti-metastatic action of these polysaccharides, triterpenoids, and polyphenols from *A. camphorata*. It appears reasonable to suggest, therefore, that *A. camphorata* metabolizes the culture medium and releases active components during the fermentation process of the submerged culture. The effective and powerful components in this extracted compound, however, still need to be characterized further.

In conclusion, our observations indicate that *A. camphorata* exerts an inhibitory effect on several essential steps of metastasis, including migration and invasion of human breast cancer cells. In addition, these extracts could regulate the activities of migration and invasion-associated proteinases and their natural inhibitors, as well as inhibit the secretion of a primary angiogenic cytokine by cancer cells. Further, here we provide clear evidence that *A. camphorata* inhibits cell invasiveness in human cancer cells through suppressing MAPK signaling pathways. As evidenced by our results, *A. camphorata* may be a powerful candidate for the development of a preventive agent for cancer metastasis. *In vivo*

studies are needed, however, to confirm the pharmacological efficacy and safety of *A. camphorata*.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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