

Full Length Research Paper

Evaluating the anti-metastatic potential of *Anisomeles indica* extract by using human oral squamous carcinoma FaDu cells

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Our previous study demonstrated that *Anisomeles indica* hexane extract (AIE) can induce cellular death in FaDu human pharynx squamous cancer cells by apoptosis. This study investigates the anti-metastatic effect of AIE on FaDu cells. First, the starting material of *A. indica* was confirmed based on nucleotide sequence analysis of ITS1, 5.8S and ITS2 of rDNA. The inhibitory effects of AIE on migration and invasion of FaDu cells were then investigated using the wound healing assay and Boyden chamber assay. Experimental results indicate that AIE significantly inhibited migration and invasion of FaDu cells in a dose-dependent manner under non-cytotoxic concentrations. Western blotting analysis revealed that AIE treatments inhibited the expression of matrix metalloproteinase-9 (MMP-9) and MMP-2 proteins dose-dependently. Moreover, according to enzyme-linked immunosorbent assay (ELISA) and gelatin zymographic assay results for the expression of MMP-9 and MMP-2, both proteins declined significantly with an increasing AIE dose. Results of this study suggest that AIE can inhibit the migration and invasion of FaDu cells by suppressing the expression of MMP-9 and MMP-2.

Key words: *Anisomeles indica* extract, FaDu pharynx cancer cells, cytotoxicity on cancer cells, cell migration and invasion, plant authentication.

INTRODUCTION

Oral cancers are generally classified as cancers of the oral cavity and of the oropharynx. Approximately, 94% of oral cancers are squamous cell carcinoma, a malignant

cancer derived from the surface epithelial cells that line the oral cavity and oropharynx (Neville et al., 2009; Rosebush et al., 2011). Oral squamous cell carcinoma (OSCC) accounts for more than 100,000 deaths worldwide annually (Gasche et al., 2011). The presence of regional metastasis, which is closely related to frequent lymph node metastasis and local invasion, in patients with OSCC is a common adverse event associated with poor prognosis and high mortality rates (Weiss, 1990; Yang et al., 2010). The treatment and control of OSCC may be improved by effective intervention before locally advanced or metastatic oral cancer develops, including during the preinvasive, intraepithelial

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Abbreviations: AIE, *Anisomeles indica* extract; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; ITS, internal transcribed spacers; MMPs, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; PBS, phosphate-buffer saline; rDNA, ribosomal DNA.

stages.

Tumor metastasis consists of a series of events, including vessel formation, cell migration, cell attachment, invasion, and proliferation (Fidler, 2005). The progress of tumor metastasis is regulated by extremely complex mechanisms. During invasion, several cytophysiological changes often occur, including loss of cell-cell adhesion along with a gain of cell-matrix adhesion, increased expression and activation of extracellular proteases to degrade the extracellular matrix (ECM) (Chen et al., 2006). Proteases frequently involved in degradation of the ECM are serine proteinase, metalloproteinases (MMPs), and cathepsins. Of these proteases, MMPs are the most essential for proteolysis of such ECM proteins as collagen, proteoglycan, fibronectin, elastin and laminin (Johnson et al., 1998). Additionally, MMPs play an important role in angiogenesis, inflammatory tissue destruction, differentiation, proliferation, and cancer cell metastasis (Chambers and Matrisian, 1997; Kajanne et al., 2007). Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the most important enzymes for degradation of the main constituent of the basement membrane, type IV collagen (Mackay et al., 1990; Nelson et al., 2000; Zucker and Vacirca, 2004).

Anisomeles indica (L.) O. Ktze is a member of the Labiatae family. As a potential and popular folk-medicine herb, *A. indica* has been prescribed for its antipyretic, carminative, antirheumatic, and analgesic activities (Lin, 2005). Scientific evidence reveals that a decoction from pre-flowering leaves and stems of *A. indica* has anti-histaminergic, free-radical scavenging, membrane stabilizing, and cyclooxygenase-I inhibitory activities. Moreover, its aqueous extract was also shown to have analgesic and anti-hyperalgesic activities (Dharmasiri et al., 2002, 2003). The ethanol extract of the whole *A. indica* plant exhibited strong anti-*Helicobacter pylori* activity (Wang and Huang, 2005) and it possessed significant anti-inflammatory activity by inhibiting the enhanced production of nitric oxide (NO) radicals, and pro-inflammatory cytokines (TNF- α and IL-12) induced by LPS/IFN- γ on murine peritoneal macrophages (Hsieh et al., 2008). Our previous study demonstrated that the hexane extract of *A. indica* can induce cell apoptosis of FaDu human pharynx squamous cancer cells by down-regulating the expressions of Bcl-2 and Bcl-xL proteins, up-regulating the expressions of Bax and Bak proteins, and activating caspase-9 and caspase-3 (Hsu et al., 2012). In Taiwan, *A. indica* is consumed for both its medicinal purposes in herbal products and as a food in some traditional dishes (Chau and Wu, 2006). A decoction of the stems of *A. indica* is commonly used as tea or juice to treat inflammatory diseases (Hsieh et al., 2008).

Previous studies on the anticancer activities of *A. indica* focused only on suppression of cancer cell proliferation, while the effect of *A. indica* extract on migration and invasion of cancer cells has not been investigated. This

work examines the inhibitory effects of *A. indica* hexane extract (AIE) on the motility and invasion of FaDu cells, which comprise a human oral squamous cell carcinoma with a high metastasis capability. The effect of AIE on the expression of MMP-2 and MMP-9 is also examined.

MATERIALS AND METHODS

Plant and chemicals

The raw materials of *A. indica* were bought from a local herb store in Kaohsiung City and were deposited in the Herbarium of I-Shou University (Kaohsiung City, Taiwan). The nucleotide sequences of internal transcribed spacers 1 (ITS1), 5.8S and ITS2 of rDNA from this plant were deposited in GenBank under the accession number JN987229. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Grand Island, New York, USA). RNase A was purchased from Gentra Systems Inc. (Minneapolis, MN, USA). Antibodies against MMP-2 and MMP-9 were from Cell Signaling Technologies (New York, USA). Anti-actin antibody was purchased from Santa Cruz Biotechnology (Delaware, CA, USA). All other chemicals were of reagent or analytical grade.

Authentication of plant material

DNA isolation, polymerase chain reaction (PCR) and DNA analysis were conducted in triplicates as described in previous study (Chiou et al., 2007). The primer set used to amplify the ITS region was 5'-GCGAGAAGTCCACTAAAC-3'/5'-GACGCTTCTCCAGACTACAAT-3'. The PCR products were examined on a 1.2% agarose gel and were sequenced. The sequences thus obtained were analyzed using DNAMAN software (Lynnon Corporation, Quebec, Canada) and were blasted with GenBank databases.

Preparation of *A. indica* extract

Dry whole plant of *A. indica* (17.8 kg) were crushed and extracted three times with 4-fold volume of 95% ethanol. After filtration by medicinal gauze, the filtrates were collected, and concentrated with a vacuum evaporator. The dry weight of this ethanol extract was 809 g, and the yield was around 4.5%. This ethanol extract (715 g) was further extracted by *n*-hexane and was dried by a freeze-dryer. The dry weight of this *A. indica* hexane extract (AIE) was 270.8 g, thus the yield of AIE from ethanol extract was 37.9%.

Cell culture and proliferation assay

Human pharynx squamous cancer cell line FaDu was purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). This cell line was grown in DMEM supplemented with 10% (v/v) inactivated FBS, 1% penicillin/streptomycin, 0.01% L-glutamine and 0.02% sodium bicarbonate, pH 7.2 to 7.4. The cells were cultivated at 37°C with 5% CO₂ and 95% air and in 100% relative humidity. Proliferation of the cells was measured according to our previous paper (Hsu et al., 2012). The viability value of each test sample was measured from five replicate experiments.

Cell migration by wound healing assay

The FaDu cells were seeded in 24-well plates at 5×10^4 cells/well

and were grown for 24 h in 500 μ l DMEM. The monolayer was artificially injured by scratching across a plate using a P1000 sterile pipette tip. Wells were washed 2 times with phosphate-buffer saline (PBS) to remove detached cells and cell debris. The DMEM with increasing AIE concentrations (0, 5, 15, and 25 μ g/ml) were added, and cells were then incubated. Images of scratched areas under each condition were examined at 0, 24, 48, and 72 h using an inverted phase-contrast microscope (Nikon Eclipse TS100, Japan) at a magnification of 10 \times . All wound healing assays were performed in triplicate.

Cell migration and invasion assays by Boyden chamber

Cell migration and invasion were assessed using the CytoSelect™ 24-well cell migration and invasion assay kits (Cell Biolabs, San Diego, CA, USA). To determine the effect of AIE on cell migration, 10⁶ cells/ml were seeded in serum-free DMEM onto a polycarbonate membrane insert (8 μ m pore size). Cells were then treated with 5, 10, 15 or 20 μ g/ml AIE. The bottom chamber was filled with DMEM containing 10% FBS as a chemo-attractant. The apparatus was incubated at 37°C for 24 h, non-migrating cells in the upper chamber were wiped away using a cotton-tipped swab, and invading cells were fixed with methanol, stained with 0.2% crystal violet, and photographed with a phase-contrast microscope at a magnification of 10 \times . Each insert was transferred to an empty well, 200 μ l of extraction solution were added, and the well was incubated for 10 min on an orbital shaker. Cell density was then measured at 560 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA). The same experimental procedure was carried out in all cell invasion experiments, except that the polycarbonate membrane was coated with a uniform layer of collagen. The inhibition effects are expressed as IC₅₀ value, which is the concentration of AIE that cause 50% inhibition of cell migration or invasion, averaged from five replicate experiments, and was obtained by plotting the percentage inhibition versus AIE concentration.

Preparation of whole-cell lysates

The cells were rinsed with iced-cold PBS for 2 times and were scraped with 150 μ l of cold modified RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% sodium deoxycholate, 1% tert-octylphenoxy poly(oxyethylene)ethanol (IGEPAL), 2 μ l of 0.25 M sodium vanadat, 20 μ l of 0.1 M ethylene glycol tetraacetic acid (EGTA), 10 μ l of 0.1 M phenylmethylsulfonyl fluoride (PMSF), 2 μ l of 5 mg/ml aprotinin, 2 μ l of 5 mg/ml leupeptin, and 2 μ l of 0.5 M ethylenediaminetetraacetic acid (EDTA)) for 20 min. The supernatants were collected by centrifugation at 10,000 \times g for 5 min at 4°C, and were used as the whole-cell lysates.

Western blotting assay

To analyze MMP-2 and MMP-9, 1.5 \times 10⁶ FaDu cells were seeded into 10-cm culture dishes with or without AIE, and were incubated for 24 h. The medium was removed and cells were washed several times with PBS (0.01 M, pH 7.2). Whole-cell lysates were prepared using the procedures described earlier. The harvested protein concentration was measured using a protein assay kit (Bio-Rad Labs). The same amounts of proteins from each extract were applied to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a nitrocellulose membrane (Immunobilon P; Millipore, Billerica, MA, USA), and then were blocked for 1 h using 10% skim milk in water. After washing in PBS containing 0.1% Tween-20 for 3 times, a

suitable dilution of specific primary antibodies were added. Following incubation overnight at 4°C, primary antibodies were washed away and secondary antibodies were added for 1 h incubation at room temperature. Finally, enhanced chemiluminescence (ECL) plus western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK) were applied to develop the signal of the membrane.

MMP-2 and -9 activities assay by ELISA kits and gelatin zymography

The FaDu cells were seeded in a 10-cm dish at 1.5 \times 10⁶ cells and were grown for 24 h under treatment with various AIE concentrations (0, 2.5, 5, and 10 μ g/ml). The medium was harvested and clarified by centrifugation to remove cells and debris. The clarified medium was concentrated using CentriconVR Centrifugal Filters with Ultracell YM-30 membranes (Millipore) at 4°C. The activities of MMP-2 and -9 were first assayed using ELISA kits (R&D Systems, Human MMP-2 and -9 Quantikine ELISA kits, Minneapolis, MN, USA). The prepared sample was subjected to electrophoresis on 8% SDS-PAGE containing 0.1% gelatin. After electrophoresis, gels were washed 2 times with 2.5% Triton X-100 and then incubated in developing buffer (50 mM Tris-HCl, 5 mM CaCl₂ and 0.2 M NaCl, pH 8.0) for 24 h at 37°C. The gel was stained with Coomassie brilliant blue R-250, followed by destaining with methanol/acetic acid/water (40/10/50, v/v/v).

Statistical analysis

All experiments were conducted for three to five independent replicates. The data are expressed in terms of mean and standard deviation. Statistical differences from the respective controls were analyzed by Student's t test with a significance level of $p < 0.05$ to 0.001 for each paired experiment. The experimental data were analyzed using Microsoft Excel software (Microsoft Software Inc., USA).

RESULTS

DNA sequence identification of *A. indica*

The dried material of *Sigesbeckia orientalis* L. is frequently misused as *A. indica* in Taiwan (Kuo, 2007). Therefore, it is critical to confirm that *A. indica* is the starting material. Figure 1 shows the nucleotide sequences of ITS1 and ITS2 of rDNA from *A. indica* and *S. orientalis*, which were deposited in GenBank under accession numbers JN987229 and JN987228, respectively. The sequence similarity of ITSs between *A. indica* and *S. orientalis* is 61.83%, indicating that this approach can effectively differentiate *A. indica* and *S. orientalis*. Blast search on the NCBI's database revealed an ITS sequence data of *A. indica* (GU726292). Twelve single nucleotide polymorphisms in ITS sequences were observed in sequence alignment of JN987229 and GU726292.

Effect of AIE on the viability of FaDu cells

The inhibitory effects of AIE on the proliferation of FaDu

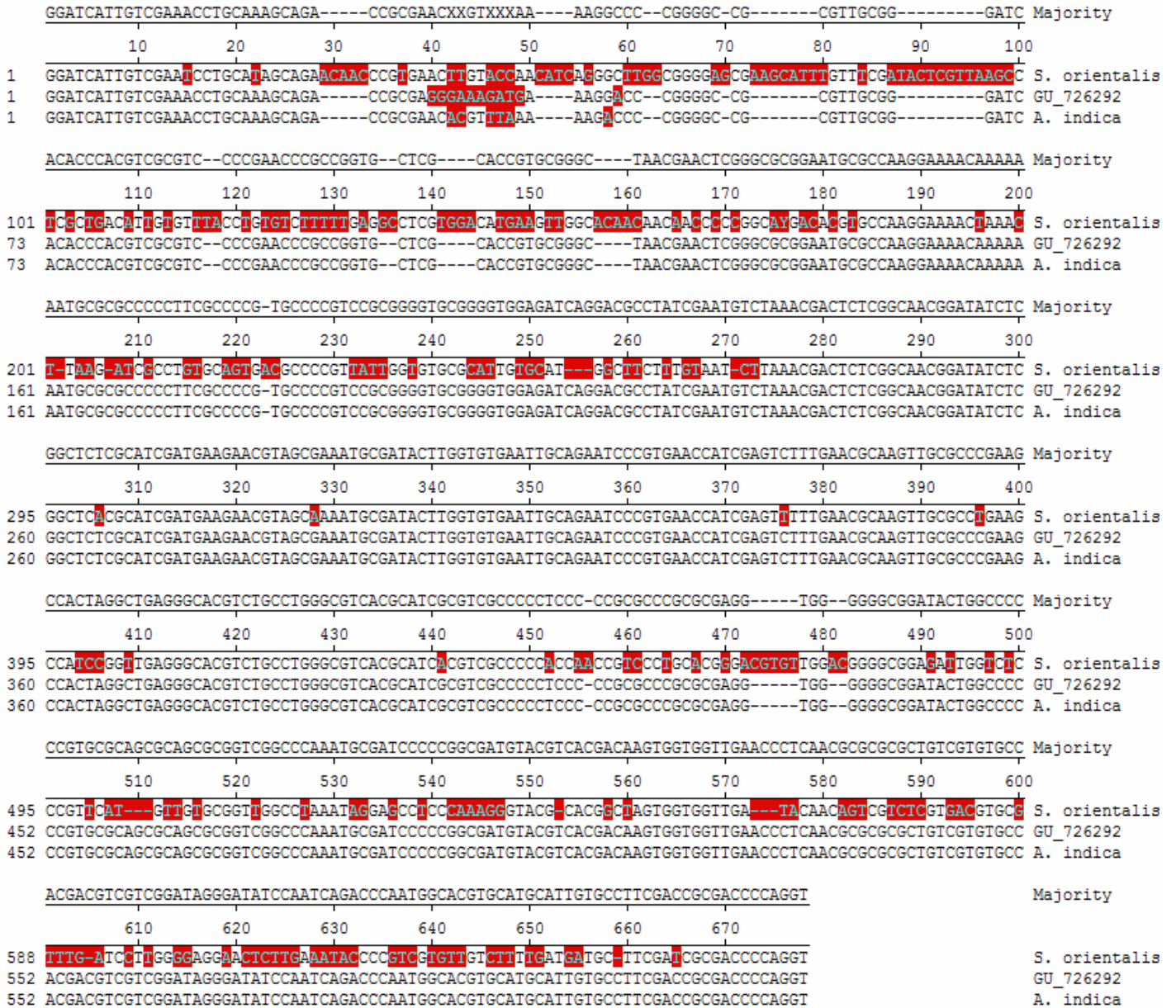


Figure 1. Comparison of the ITS nucleotide sequences of rDNA of *A. indica* (JN987229), *S. orientalis* (JN987228) and other sequences obtained from the NCBI's database of *A. indica* (GU726292). The sites exhibiting variance are shaded in red.

cells were examined and quantified using the MTT assay. The AIE exhibited a dose- and time-dependent inhibitory effect on the growth of FaDu cells (Figure 2). However, cell growth was not significantly altered by 24 h treatment with AIE concentrations of 0 to 20 µg/ml. The percentage of viable cells exceeded 80% as compared to that of the control (DMSO-treated alone). Changes in cell viability in this concentration range were considered modest and insufficient to account for the significant decrease in cell migration and invasion under these treatment conditions. Thus, this AIE concentration range was utilized in all subsequent experiments.

AIE-induced inhibition of migration of FaDu cells

The effects of AIE on cell migration were first examined using the wound healing assay and results are as shown in Figure 3. When not treated with AIE, cells moved rapidly into the scratched area, and the number of migrating cells increased over time. Conversely, the cells exposed to high AIE concentrations and long incubation times had a reduced ability to migrate and fill the wound area, as compared with the control cells. These results indicate that AIE inhibited migration of FaDu cells in a dose- and time-dependent manner.

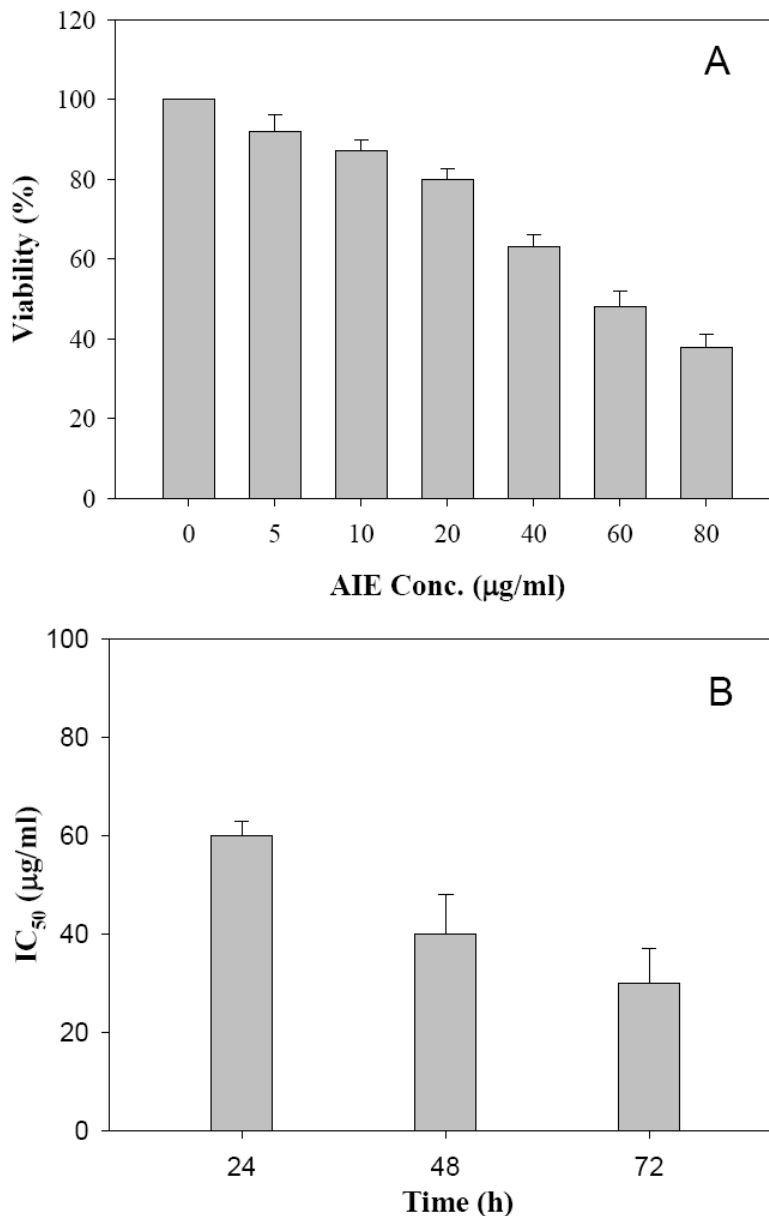


Figure 2. Effects of AIE on FaDu cell proliferation. (A) Variation in viability under treatment with DMSO only (AIE conc. = 0 µg/ml), or with different AIE doses for 24 h treatment. (B) Variation in IC₅₀ values under different treatment durations. Data were estimated from five replicate experiments.

A cell migration assay with the Boyden chamber was applied to examine further the effect of AIE on the motility of FaDu cells. Cells were plated in the upper chamber, and the number of cells that moved to the underside of the coated polycarbonate membrane was counted under a light microscope. The number of cells that migrated to the lower chamber was reduced significantly by AIE treatment (Figure 4). The AIE retarded the migration of FaDu cells dose-dependently when compared with that of untreated controls. Motility decreased 62% ($p < 0.001$) after treatment with 20 µg/ml AIE for 24 h. The IC₅₀ value

was estimated to be 16.0 µg/ml.

AIE-induced inhibition of invasion of FaDu cells

The inhibitory effect of AIE on the invasion of FaDu cells was investigated using a cell invasion assay with the Boyden chamber coated with collagen. The number of cells that invaded from the upper to the lower chamber was significantly inhibited by AIE treatment (Figure 5). Quantification of cells in the lower chamber indicates that

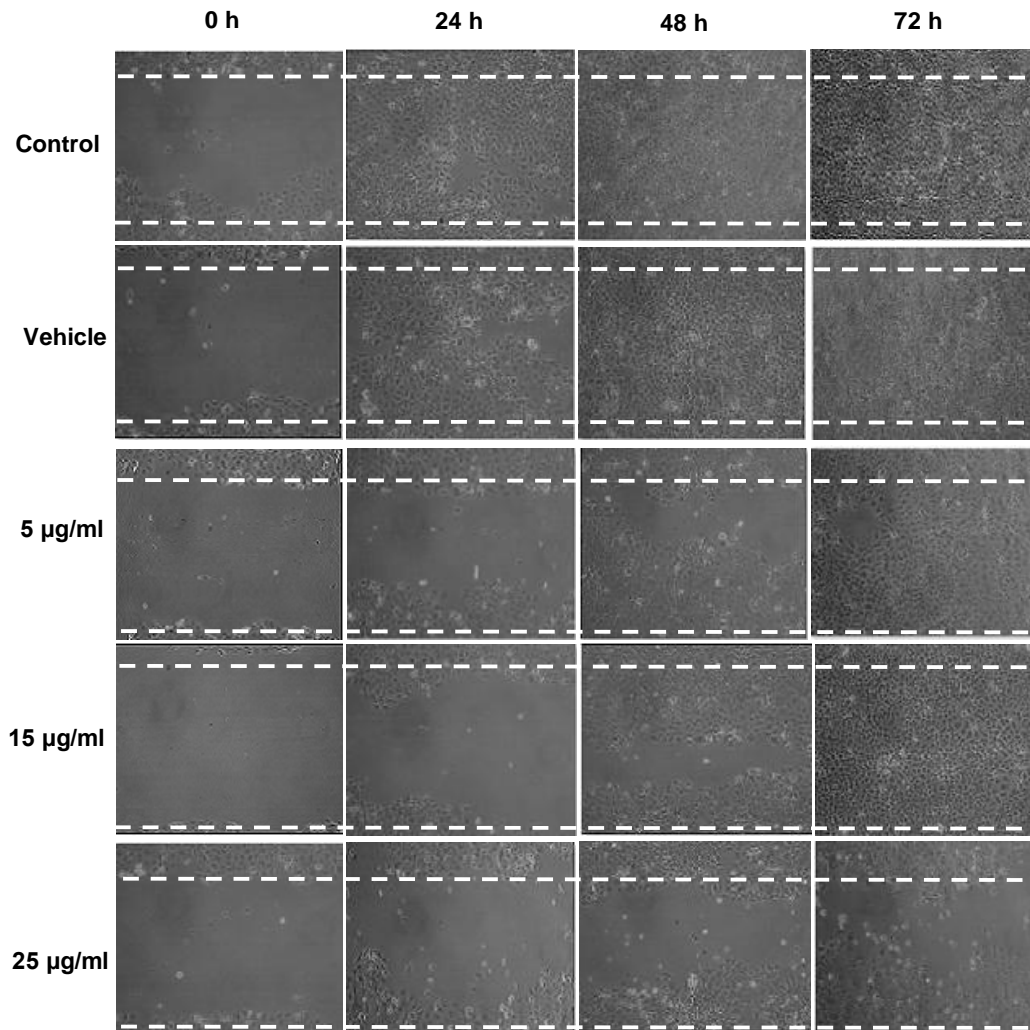


Figure 3. Effects of different AIE doses on FaDu cell motility. Monolayers of FaDu cells were wounded by scratching with a pipette tip, and were incubated under treatment with experimental media only (control), DMSO only (vehicle), or with different AIE doses for different durations. Photomicrographs were taken directly from culture plates with a phase-contrast microscope (magnification 10 \times).

AIE treatment significantly inhibited FaDu cell invasion in a concentration-dependent manner. Invasion was reduced to 67.9% ($p < 0.001$) and then roughly 50% ($p < 0.001$) after treatment with 10 $\mu\text{g/ml}$ AIE and 20 $\mu\text{g/ml}$ AIE for 24 h, respectively. The IC_{50} value was estimated to be 18.9 $\mu\text{g/ml}$.

Effects of AIE on protein expression of MMPs in FaDu cells

The protein levels and activities of MMP-2 and MMP-9 were determined by western blotting, the ELISA, and the gelatin zymographic assay. Western blot analysis demonstrates that AIE treatment reduced the protein level of MMP-9 (92 kDa) in a dose-dependent manner (Figure 6A). In contrast, the protein level of MMP-2 (72

kDa) was not affected significantly. By the ELISA, the activity of MMP-9 decreased dramatically as the AIE concentration increased, while that of MMP-2 changed only minimally (Figure 6B). Similar analytical results for the effects of AIE on the activities of MMP-9 and MMP-2 in FaDu cells were obtained using the gelatin zymographic assay (Figure 6C).

DISCUSSION

The authenticity of Chinese medicinal plants is the main concern in ensuring safety and therapeutic efficacy, especially for species that share a name or have a similar appearance (Heubl, 2010). Plant species are generally categorized based on appearance, as well as on tissue slides of flowers, stems, roots, and seeds. However,

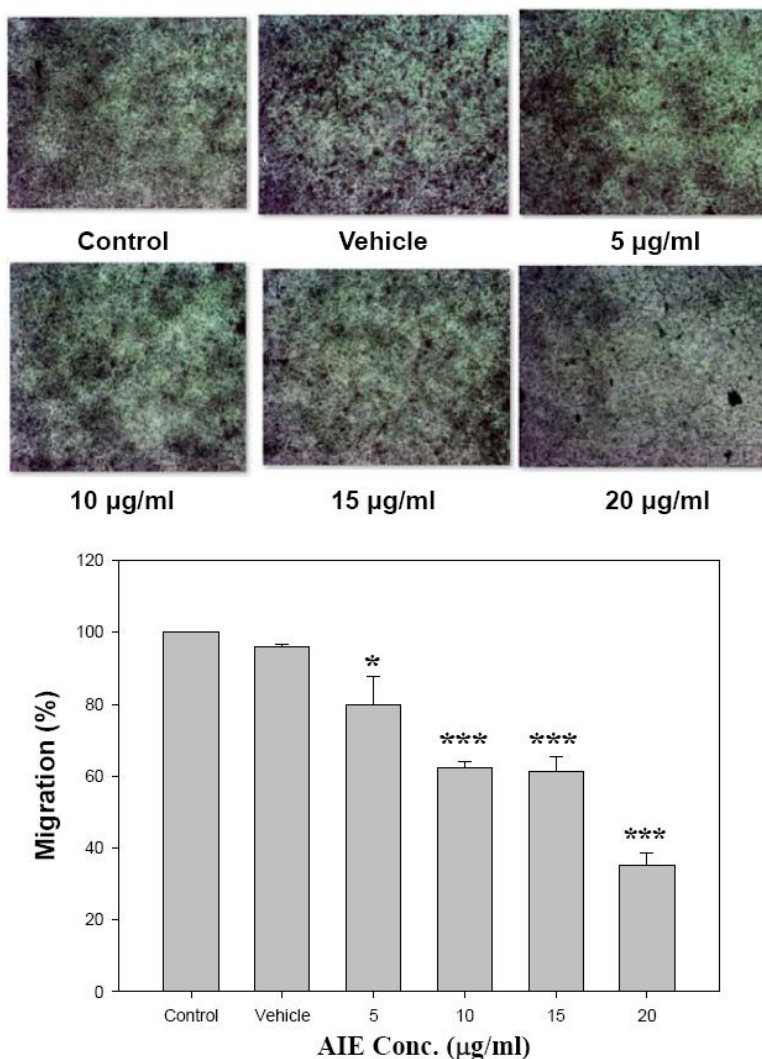


Figure 4. Effects of AIE on FaDu cell migration *in vitro*. The migration assay was performed using the Boyden chamber. The FaDu cells were treated with various concentrations of AIE for 24 h. The cells penetrated through the polycarbonate membrane to the lower surface of the filter were stained with crystal violet, photographed with a phase-contrast microscope (magnification 10 \times), and quantified. Data were estimated from five independent experiments. Statistical significance of differences between AIE-treated groups and the control (AIE conc. = 0 $\mu\text{g/ml}$) were quantified by the Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

morphological and histological characterization of herbal medicines often fails to distinguish closely related species (Shaw et al., 2002). Recently, recognition of plants using DNA barcodes has attracted significant attention (Chen et al., 2010; Ebihara et al., 2010). In this study, the variation in the ITSs sequences between *A. indica* and *S. orientalis* was used to clarify the authentic materials (Figure 1). Twelve single nucleotide polymorphisms in ITS sequences of *A. indica* (JN987229 and GU726292) might result from PCR errors or minor gene mutation among different individuals within a species (Gonzalez et al., 2009).

This study is the first to investigate the anti-metastatic effect of AIE on FaDu cancer cells. Our previous study demonstrated that AIE can induce cell death in FaDu cells, mainly by the apoptotic effect and partly by the necrotic effect, and was associated with cell-cycle arrest (Hsu et al., 2012). Notably, OSCC is the most malignant neoplasm in the oral cavity, and causes painful localized inflammation, significant local invasion and distant metastatic effects. Since *A. indica* has strong anti-inflammation and anti-proliferation activities, if it exerts anti-metastatic activity on cancer cells, it may become a good complementary agent for treating patients with oral

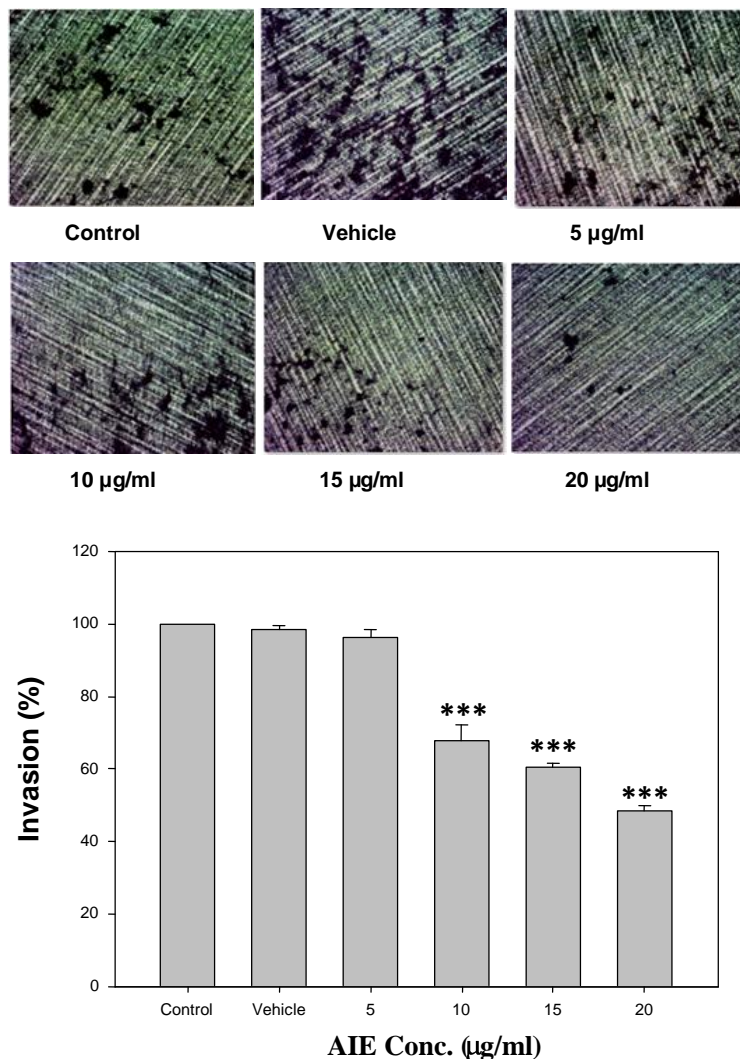


Figure 5. Effects of AIE on FaDu cell invasion *in vitro*. The invasion assay was performed using the Boyden chamber. The FaDu cells were treated with various concentrations of AIE for 24 h. The cells penetrated through the matrigel membrane to the lower surface of the filter were stained with crystal violet, photographed with a phase-contrast microscope (magnification 10 \times), and quantified. Data were estimated from five independent experiments. Statistical significance of differences between AIE-treated groups and the control (AIE conc. = 0 $\mu\text{g/ml}$) were quantified by the Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

cancers. The cancer cell metastatic process consists of multiple events involving cell motility, cell invasion, surface adhesion, and degradation of extracellular matrix (ECM) (Fidler, 2005). To demonstrate that AIE affects cell migration and invasion, this study excluded the effect of AIE on tumor cell growth by MTT assay, indicating that cell viability was not altered significantly by AIE treatment at concentrations of 0 to 20 $\mu\text{g/ml}$ (Figure 2). This dose range was applied in all subsequent experiments to eliminate the influence of cell growth on observed parameters. The AIE treatment was shown to inhibit the migration and invasion potential of FaDu cells by wound

healing analysis (Figure 3) and the Boyden chamber assay (Figures 4 and 5) in a dose- and time-dependent manner. Tumor invasion requires degradation of basement membranes, proteolysis of the ECM, pseudopodial extension, and cell migration. The basement membrane, which separates epithelial and mesenchymal cell compartments, is made of matrix macromolecules, such as type IV collagen, laminin, and heparan sulfate proteoglycans, and is the first barrier of the ECM against cancer invasion (Lim et al., 2008). Therefore, a Boyden chamber with a polycarbonate membrane coated with a collagen layer was used in this study to examine the

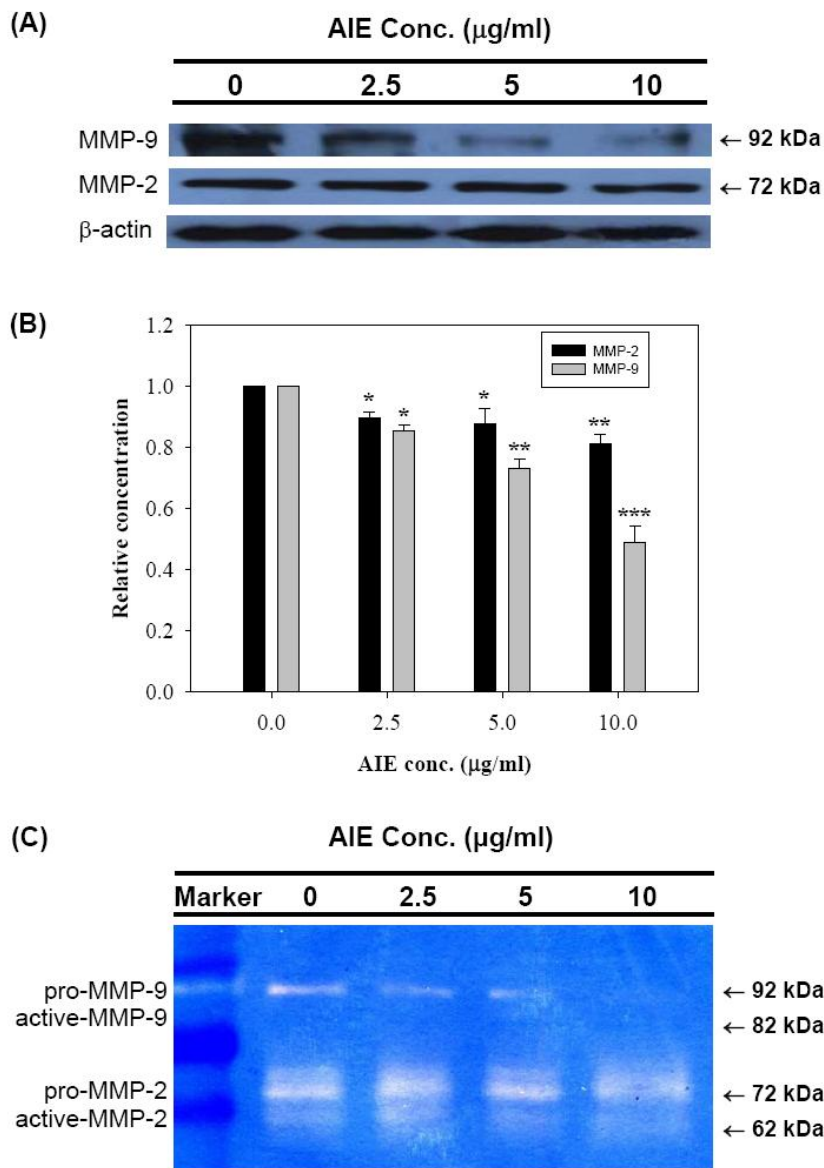


Figure 6. Effects of AIE on MMP-2 and MMP-9 expressions in FaDu cells. (A) Expressions of MMP-2 and MMP-9 were analyzed by Western blotting. (B) Quantitative MMP-2 and MMP-9 activity was assessed by ELISA. Data were estimated from five independent experiments. Statistical significance of differences between AIE-treated groups and the control (AIE conc. = 0 $\mu\text{g/ml}$) were quantified by the Student's *t*-test (**p* < 0.05; ***p* < 0.01; ****p* < 0.001). (C) Activities of MMP-2 and MMP-9 analyzed by gelatin zymographic assay. The upper band corresponds to the proform gelatinase, the lower band represents the activated form.

inhibitory effect of AIE on invasion of FaDu cells.

Several enzymes that degrade basement membrane, such as MMPs, are assumed to play an important role in the cancer invasion and metastatic processes. Particularly, activated forms of MMP-2 and MMP-9 have been identified to be important in cancer invasion and metastasis due to their ability to cleave type IV collagen. Studies have proved that the expressions of these two

enzymes are closely associated with nodal and distant metastasis and the recurrence rate for patients with oral cancers (Yoshizaki et al., 2001; Kunigal et al., 2007; Li and Wu, 2010). Researchers have recently focused on identifying bioactive compounds from natural sources that can suppress the expression of MMP-2 or -9 and, thus, these compounds would be potential candidates for cancer prevention and treatment, including those for

patients at high risk of developing oral cancer (Hwang et al., 2011). In this study, the association between AIE treatment and expressions of MMP-2 and -9 in FaDu cells was examined by western blotting, the ELISA, and the gelatin zymographic assay (Figure 6). Experimental results show that AIE markedly down-regulated MMP-9 activity in a dose-dependent manner, and caused a relatively minor change to MMP-2 activity. These experimental results suggest that the anti-metastatic effect of AIE was associated with inhibition of enzymatic degradation processes of tumor metastasis.

Conclusively, AIE can significantly inhibit the migration and invasion of highly metastatic FaDu cells, mainly by suppressing MMP-9 and MMP-2 expressions, suggesting that AIE is a potential complementary agent for the prevention or treatment of cancer metastasis. Further studies are still needed to elucidate the detailed pathway.

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