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Full Length Research Paper

Cytotoxic effect of *Anisomeles indica* extract on human pharynx squamous cancer cells

Hsia-Fen Hsu[1], Shih-Fan Chang[2], Zi-Hua Chen[2], Shyng-Shiou F. Yuan[3], Yu-Duan Tsai[2,4], Chao-Ping Wang[5], Shih-Wei Wang[2,6], Li-Wen Fang[1] and Jer-Yiing Houng[1,2]**

[1]Department of Nutrition, I-Shou University, Kaohsiung City, Taiwan, Republic of China.
[2]Institute of Biotechnology and Chemical Engineering, I-Shou University, Kaohsiung City, Taiwan, Republic of China.
[3]Department of Obstetrics and Gynecology, E-DA Hospital, I-Shou University, Kaohsiung City, Taiwan, Republic of China.
[4]Department of Neurosurgery, E-DA Hospital, Kaohsiung City, Taiwan, Republic of China.
[5]Division of Cardiology, Department of Internal Medicine, E-DA Hospital, Kaohsiung City, Taiwan, Republic of China.
[6]Division of Allergy, Immunology, and Rheumatology, Department of Internal Medicine, E-DA Hospital, Taiwan, Republic of China.

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This study attempted to investigate the inhibitory effect of *Anisomeles indica* hexane extract (AIE) on the proliferation of FaDu human pharynx squamous cancer cells and the apoptotic effect of this extract on FaDu cells. AIE exhibited significant cytotoxicity toward FaDu cells in a dose- and time-dependent manner (IC_{50} = 60.1 µg/ml for 24 h treatment and 29.7 µg/ml for 72 h treatment). In comparison, AIE displayed lower sensitivity to normal lung fibroblast MRC-5 cells (IC_{50} = 102.7 µg/ml for 24 h treatment). Typical morphological changes of apoptosis, such as cell shrinkage, rounding, apoptotic vacuoles and forming majority of the floating cells, were observed microscopically when the FaDu cells were treated with AIE. Cytometric analysis demonstrated that FaDu cells were arrested at the G2/M phase. Moreover, AIE induced FaDu cell death mainly via the apoptosis pathway and partly via the necrosis pathway. The extract induced FaDu cell apoptosis by down-regulating Bcl-2 and Bcl-xL protein expression, up-regulating Bax and Bak protein expression, and activating caspase-9 and caspase-3. This investigation suggests that AIE possesses potential anticancer activity through growth inhibition *via* cell cycle arrest and apoptosis of FaDu cells.

Key words: *Anisomeles indica* (L.) O. Ktze extract, FaDu pharynx squamous cancer cells, cytotoxicity, cancer cells, apoptosis.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common head and neck cancer. Oral and pharyngeal cancer ranks together as the sixth most common cancer worldwide (Warnakulasuriya, 2009). In Taiwan, the incidence and mortality of oral cancer death have been steadily growing during the last decade. Approximately 9.7 persons per 100,000 of population die annually of oral cancer, and oral cancer has become the sixth and the fourth most frequent cause of cancer death in the total and male populations, respectively (Department of Health, 2009). Considerable evidence indicates that complementary and alternative medicine use in Western society has increased steadily. Population-based surveys have provided estimates of increased use and demand for complementary and alternative medicine products in the United Kingdom and th e United States. This trend is

*Corresponding author. E-mail: jyhoung@isu.edu.tw. Tel: +886-7-6151100. Fax: +886-7-6155150.

Abbreviations: AIE, *Anisomeles indica* extract; **DMSO,** dimethyl sulfoxide; **5-FU,** 5-fluorouracil; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **OSCC,** oral squamous cell carcinoma; **PBS,** phosphate-buffer saline; **PI,** propidium iodide.

particularly clear in the field of cancer and palliation (Chatwin and Tovey, 2004; Shakeel et al., 2008). Recently, traditional medicine has rapidly integrated with modern medicine, and the potential for using traditional herbs in cancer treatment has received considerable attention. Herb extracts, which can abate or reverse cancer development and/or progression, are frequently administered together with conventional cancer therapies to increase survival rates and improve patient quality of life (Kinghorn et al., 2003; Mohan et al., 2010a).

Anisomeles indica (L.) O. Ktze (former name: *A. indica* (L.) Kuntze) belongs to the Labiatae family which is a traditional Chinese medicinal herb that possesses antipyretics, carminative, antirheumatic and analgesic activities (Lin, 2005). Chinese have long used *A. indica* to make herbal tea, with the tea being popularly known as Yu-Chen-Tsao tea. In Taiwan, *A. indica* has been used as a folk medicine in treating inflammatory skin diseases, liver disease and protection, gastrointestinal disease, hypertension and immune system deficiencies (Kao, 1985). Based on the scientific evidence, the aqueous extract of *A. indica* was shown to have analgesic and anti-hyperalgesia activities (Dharmasiri et al., 2002, 2003). The ethanol extract and methanol extract exhibited strong anti-*Helicobacter pylori* activity (Wang and Huang, 2005) and anti-inflammatory activities (Hsieh et al., 2008; Rao et al., 2009), respectively. Moreover, macrocyclic diterpenoids isolated from whole plants of *A. indica* showed anti-HIV effect (Shahidul Alama et al., 2000) and anti-proliferative activity against KB human nasopharynx cancer cells (Arisawa et al., 1986). Furthermore, studies have also examined the suppression of cell proliferation of *A. indica* extracts on several human cancer cell lines: Colon 205 (colon), PC 3 (prostate), HepG2 (hepatoma) and MCF 7 (breast) (Hsieh et al., 2008). However, to our knowledge, the anticancer activity and its actions on OSCC of *A. indica* extract has not yet been clarified well.

OSCC is a malignant carcinoma that causes significant and painful localized inflammation. Since *A. indica* possesses strong anti-inflammatory activity, if it exerts anti-proliferation activity on cancer cells, it may become a good complementary treatment agent for oral cancer patients. This study thus investigates the feasibility of the hypothesis that *A. indica* extract inhibits cell growth and induces cell apoptosis of FaDu cancer cells.

MATERIALS AND METHODS

The raw material of *A. indica* was purchased from a local herb store in Kaohsiung City and deposited in the Herbarium of I-Shou University (Kaohsiung City, Taiwan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Grand Island, New York, USA), RNase A was purchased from Gentra Systems Inc. (Minneapolis**,** MN, USA), while annexin V-FITC apoptosis detection kit was obtained from Strong Biotech Co. (Taipei, Taiwan). Antibodies against procaspase-3, -8, -9, Bcl-2, Bcl-xL, Bax, Bad and Bak were purchased from BD Bioscience (Franklin Lakes, NJ, USA).Anti-β-actin antibody was from Santa Cruz Biotechnology (Delaware, California, USA), and 5-Fluorouracil 5-FU) was from (Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of reagent or analytical grade.

Extraction of plant material

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 separated into several fractions using the partition procedure with *n*-hexane, ethyl acetate (EA) and *n*-butanol, and dried by a freeze-dryer. The overall yield of these dried *A. indica* extracts were 1.8, 1.7 and 0.1%, respectively.

Cell culture and proliferation assay

Human pharynx squamous cancer cell line FaDu and human normal lung fibroblast cell line MRC-5 were purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). These two cell lines were grown in DMEM supplemented with 10% (v/v) inactivated fetal bovine serum, 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% CO_2 and 95% air, under 100% relative humidity. FaDu cells were seeded at a density of 1×10^4 cells/well in 96-well plates containing medium with various concentrations of *A. indica* extract. After various durations of cultivation, the medium solution was removed. An aliquot of 100 µL of medium containing 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was loaded to each well of the plate. The cells were cultured for 2 h, and then the medium solution was removed. An aliquot of 100 µL of DMSO ((dimethyl sulfoxide) was added to the plate, which was shaken until the crystals dissolved. Cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 570 nm in a microplate ELISA (Enzyme-linked immunosorbent assay) reader (Model 550, Bio-Rad Laboratories, Hercules, CA, USA). Cytotoxicity of each sample was expressed as IC_{50} value. The value is the concentration of test sample that cause 50% inhibition of cell growth, averaged from three to five replicate experiments, and was obtained by plotting the percentage inhibition *versus* concentration of test sample. 5-FU was used as the positive control which is an effective anticancer drug. Control experiments received an identical volume of solvent DMSO (final concentration of 0.5%).

Cell morphological examination

FaDu cells at a density of 1×10^4 cells/well grown on 24-well plates were treated with *A. indica* hexane extract (AIE) at different doses of 0, 45, 60 and 75 µg/ml for 24 and 48 h. The morphological changes were observed under an inverted phase contrast microscope (Nikon Eclipse TS100, Japan) at 40× magnifications. Cell shrinkage, rounding, partial detachment and the lobulated appearance were observed as signs of apoptosis.

Cell cycle analysis

To investigate the effect of AIE on the cell cycle distribution, FaDu cells at 5×10^4 cells/well grown on 24-well plates were treated with various AIE concentrations and cultured for 48 and 72 h. The treated cells were harvested, washed with phosphate-buffer saline (PBS) and trypsinized by trypsin/ethylenediaminetetraacetic acid (EDTA) solution. After washing twice with cold PBS, cells were suspended in hypotonic solution (0.1% sodium citrate and 0.1% Triton X-100)

containing 40 µg/ml propidium iodide (PI), followed by an incubation at 4°C for 12 h. The stained cells were analyzed with flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) and the data were consequently calculated using WinMDI 2.9 software (TSRI, La Jolla, CA, USA).

Measurement of apoptotic ratio of FaDu cells

The apoptotic effects of AIE and 5-FU on FaDu cells were determined by the Annexin V-FITC staining method and flow cytometry. The cells at 5×10^4 cells/well grown on 24-well plates were treated with various AIE concentrations and cultured for 48 and 72 h. The treated cells were harvested, washed with PBS and then treated with trypsin/EDTA solution. The suspended cells were centrifuged at 300 × g for 5 min. The cell pellet was added in 100 µl of Annexin V-FITC staining-solution and incubated for 15 to 20 min at 25°C. The cells were then analyzed by flow cytometer and the data were calculated with WinMDI 2.9 software.

Western blot analysis

Western blotting was performed to detect the proteins of procaspase-9, -8, -3 and Bcl-2-family. FaDu cells of 1.5×10^6 cells were seeded onto 100 mm culture dishes with or without AIE, and were incubated for 24 h. The medium was removed and the cells were washed several times with PBS (0.01 M, pH 7.2). Following removal of the supernatant solution, the cells were lysed with lysis buffer (0.1 ml lysis buffer/each plate) for 20 min. The composition of lysis buffer was 1 ml RIPA buffer (150 mM NaCl, 50 mM Tris, 0.5% sodium deoxycholate, 1% tert-octylphenoxy poly (oxyethylene) ethanol (IGEPAL)), 4 µl of 0.25 M sodium vanadate, 20 µL of 0.1 M ethylene glycol tetraacetic acid (EGTA), 10 µl of 0.1 M phenylmethylsulfonyl fluoride (PMSF), 2 µl of 5 mg/ml aprotinin, 2 µl of 5 mg/ml leupeptin, and 2 µl of 0.5 M EDTA. The supernatants were collected by centrifugation at 10,000×g for 5 min at 4°C, and were used as the cell protein extracts. 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% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane (Immunobilin P; Millipore, Billerica, MA, USA), and then blocked for 1 h using 10% skim milk in water. After washing in PBS containing 0.1% Tween 20 for 3 times, primary antibodies against procaspase-9, -8, -3, Bcl-2, Bcl-xL, Bax, Bad, Bak or β-actin were added at a v/v ratio of 1:1000. Following overnight incubation at 4°C, the primary antibodies were washed away and the horseradish peroxidase (HRP)-linked secondary antibodies were added for 1 h incubation at room temperature. Finally, the Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK) were used to develop the signal of the membrane.

Statistical analysis

All experiments were conducted for three to five independent replicates. The data are expressed in terms of mean and standard deviation. The experimental data were analyzed using Microsoft Excel software (Microsoft Software Inc., USA).

RESULTS

Cytotoxicity of *A. indica* extracts on FaDu cancer cells

According to the results of MTT assay on FaDu cells, the IC_{50} values of AIE and EA extract for 24 h treatment were 60.1 and 108.2 µg/ml, respectively, whereas, the *n*-butanol extract showed no significant cytotoxicity on FaDu cells under 500 µg/ml. Therefore, AIE was chosen to examine in the following experiments. Figure 1 shows the cytotoxic effects of AIE at various concentrations (0 - 100 µg/ml) and treatment times (24 to 72 h) on FaDu cells, and on the normal lung fibroblast MRC-5 cells for comparison. Figure 1A shows that the viability of FaDu cells was significantly reduced by increasing the AIE concentration for 24 h treatment. Figure 1B demonstrated that the cytotoxic effect of AIE on FaDu cells was time-dependent during the examined period. For the cytotoxicity of AIE on MRC-5 cells, dose-dependent reduction of cell growth was observed with an IC_{50} of 102.7 µg/ml for 24 h treatment (Figure 1C). Thus, AIE exerted a low adverse impact on normal cells.

The changes in cell morphology were assessed at different concentrations of AIE treatment for 24 and 48 h, respectively. Compared to the control, FaDu cells treated with the experimental media (the vehicle group) appeared as healthy as the control group (Figure 2). After exposure to 45 µg/ml of AIE or higher, numbers of FaDu cells were noticeably reduced, and the cells displayed the characteristic features of shrinkage, rounding, apoptotic vacuoles, partial detachment and the lobulated appearance of apoptotic cells both in a dose- and time-dependent manner.

Effect of AIE on cell cycle kinetics

The effects of AIE on cell cycle phase distribution in FaDu cells were examined by flow cytometry under treatment with different AIE concentrations for 48 and 72 h. The experimental results shown in Figure 3 demonstrated that the number of cells increased in the G2/M phase and decreased in the G0/G1 phase, in both dose- and time-dependent manner. In comparison, 5-FU arrested FaDu cells at G0/G1 phase.

Induction of apoptosis by AIE

To confirm and quantify AIE induced apoptosis in FaDu cells, cells were stained with annexin V-FITC/PI, and then subsequently analyzed by flow cytometry. The analytical results of the flow cytometry are expressed in four quadrants (Figure 4). Generally, the dots in the lower left (LL) quadrant are viable cells; those in the lower right (LR) quadrant are cells undergoing early apoptosis; those in the upper right (UR) quadrant are cells undergoing late apoptosis; while those in the upper left (UL) quadrant are cells in necrosis. Figure 4B and 4C indicate that the FaDu cells in the control and vehicle groups died via both apoptosis and necrosis pathways. When FaDu cells were treated with AIE, the dots shifted to the LR quadrant dose and time dependently (Figure 4H), indicating that the cells

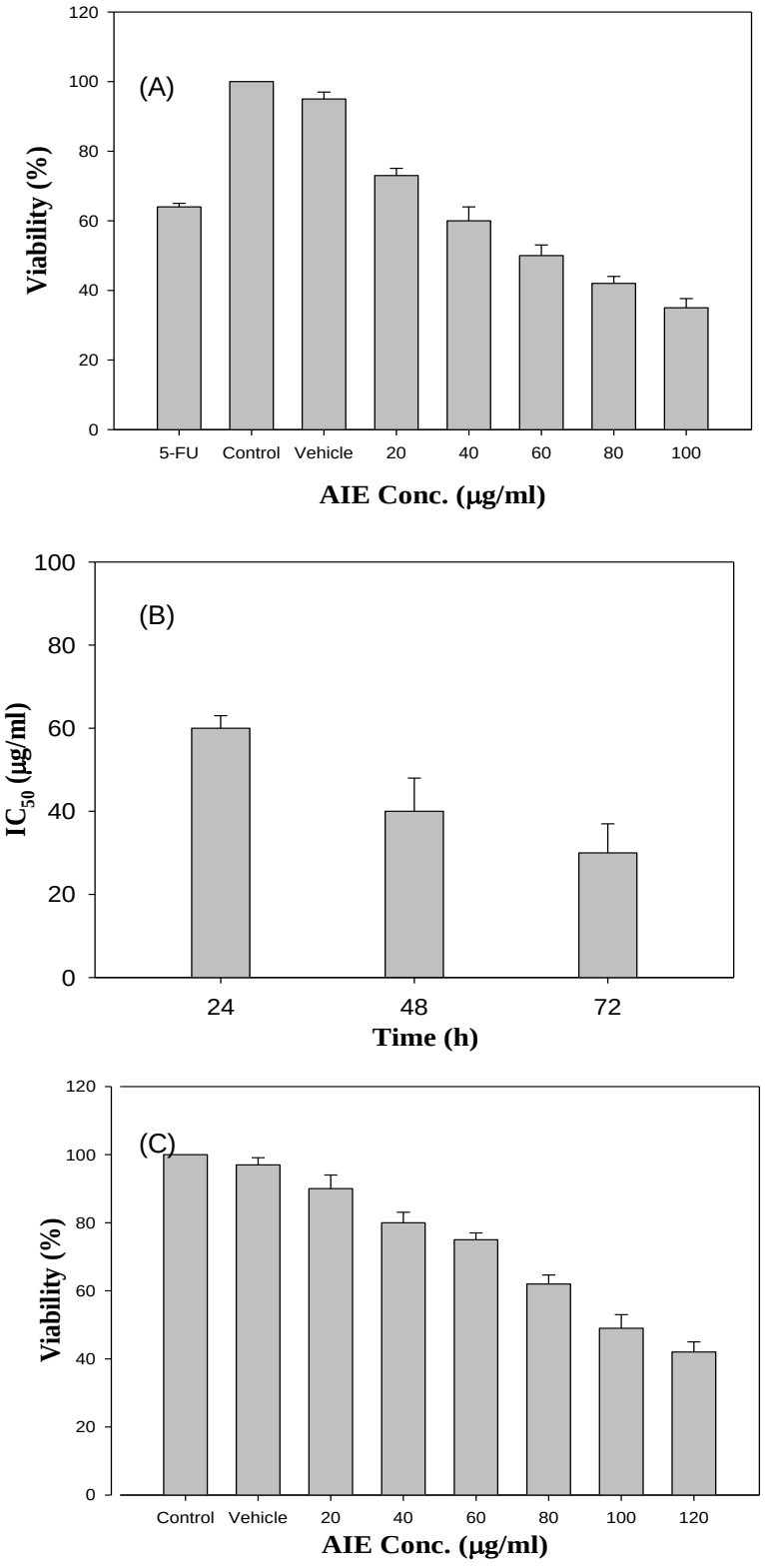

Figure 1. Effects of different AIE doses and treatment durations on proliferation of FaDu and MRC-5 cells. (A) Variation in viability under treatment with experimental media only (control), DMSO only (vehicle) or with different AIE doses on FaDu cells for 24 h. 5-FU of 100 μg/ml was used as a positive control. (B) Variation in IC_{50} values on FaDu cells under different treatment durations. (C) Variation in viability under different AIE doses on MRC-5 cells for 24 h. Data were estimated from five replicate experiments.

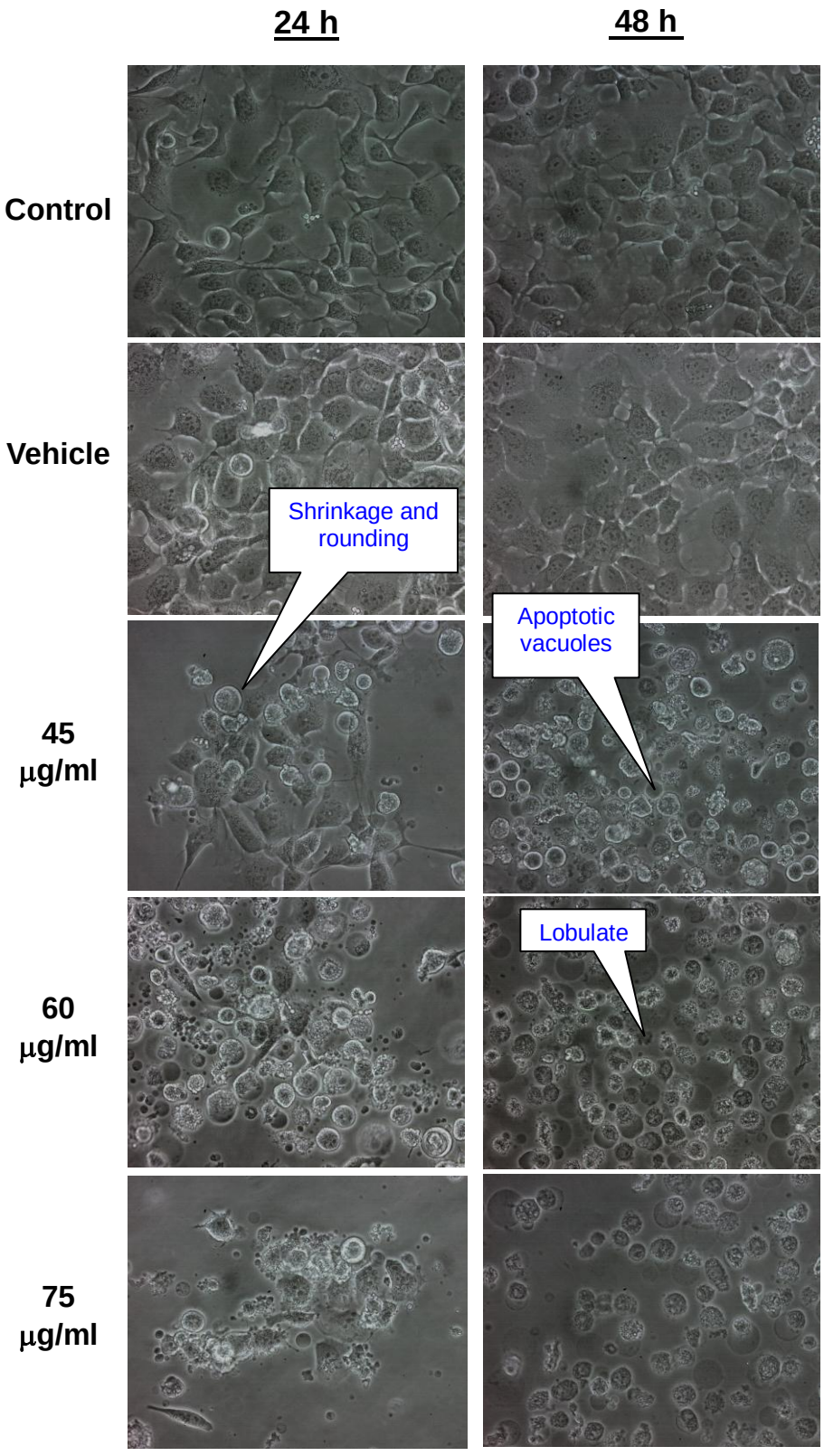

Figure 2. Microscopic observation of FaDu cells undergoing apoptosis by treating with AIE. Cells were treated in experimental media only (control), with DMSO only (vehicle) or with the culture media containing 45, 60 or 75 µg/ml AIE for 24 and 48 h. Photomicrographs were taken directly from culture plates with a phase contrast microscope (magnification 40×).

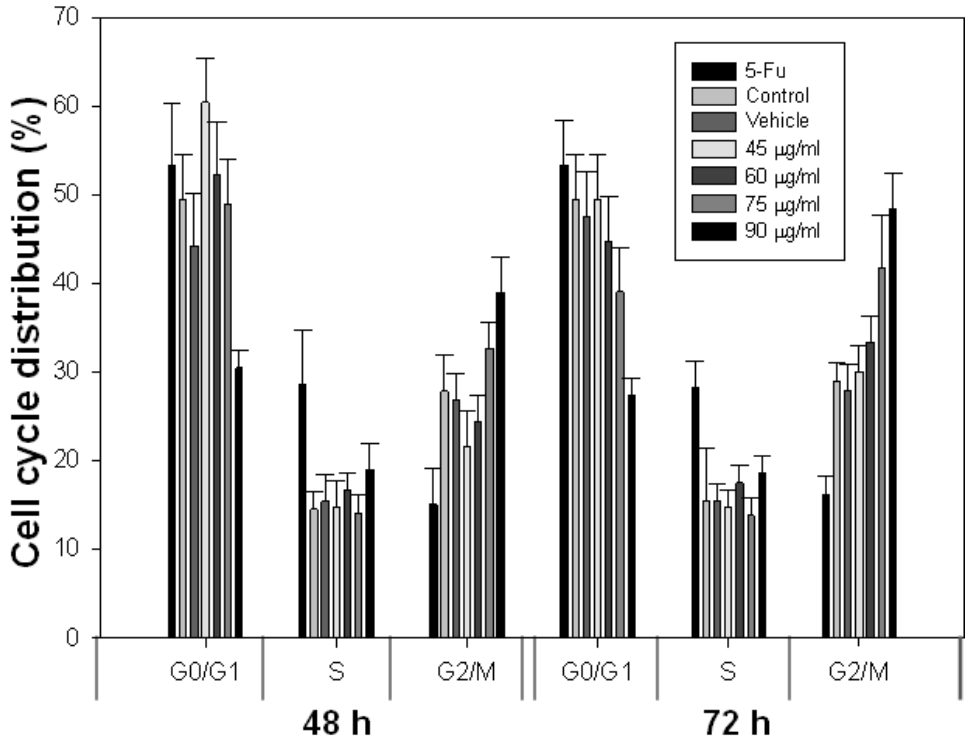

Figure 3. Cell cycle analysis of FaDu cells treated with different AIE concentrations by flow cytometry. FaDu cells at a density of 5×10^4 cells/well were incubated in experimental media only (control), treated with DMSO only (vehicle) or treated with media containing various concentrations of AIE for 48 and 72 h. The cell number percentage in each phase (sub-G1, G0/G1, S and G2/M) of the cell-cycle was analyzed by WinMDI 2.9 software. 5-FU of 100 μg/ml was used as a positive control.

moved to the early apoptotic stage. The cell population of the UR quadrant increased with the AIE dose, indicating that cells had progressed gradually to the late stage of apoptosis. These results indicated that AIE enabled the translocation of phosphatidylserine to expose on the cell surface, and hence induced early apoptotic induction in FaDu cells. Furthermore, this study also observed that some cell dots shifted to the UL quadrant, implying that a small portion of the cells died through the necrosis pathway.

Effects of AIE on the expression of apoptosis-related proteins

To further elucidate the molecular mechanism involved in apoptosis by AIE, immunoblot analysis was performed for the proteins involved in apoptosis. Treatment with AIE resulted in caspase-3 (as demonstrated by the cleavage of procaspase-3 (32 kDa precursor)) and caspase-9 (as shown by the cleavage of procaspase-9 (47 and 37 kDa precursors)) activation in a dose-dependent manner (Figure 5A). However, procaspase-8 (55 kDa precursor) expression did not vary significantly with AIE concentration. The expression of Bcl-2 family proteins under AIE treatment was further assessed in FaDu cells in which, the pro-apoptotic subgroup, including Bax, Bad and Bak, promotes cell death; while the anti-apoptotic subgroup, including Bcl-2 and Bcl-xL, inhibits apoptosis (Catz and Johnson, 2003). Figure 5B shows that Bax and Bak expression increased, while Bcl-2 and Bcl-xL protein level decreased with increasing AIE concentration. The Bad/Bcl-xL and Bax/Bcl-2 ratios in a cell regulate susceptibly to apoptosis (Vander Heiden and Thompson, 1999; Hsu et al., 2008). Figure 5C reveals that the Bax/Bcl-2 ratio increased notably with AIE concentration; meanwhile, the ratio of Bad/Bcl-xL remained unchanged, mainly due to the insignificant change of Bad protein under the AIE induction on FaDu cells.

DISCUSSION

OSCC is the most malignant carcinoma in the oral cavity (Chiu et al., 2010; Yang et al., 2010), and chemotherapy is one of the most efficient treatment approaches. Cisplatin and 5-FU are the chemotherapeutic agents frequently used to treat head and neck cancers; however, the response rate is typically only 20 to 30% in patients treated for relapsed or recurrent disease (Jacobs et al.,

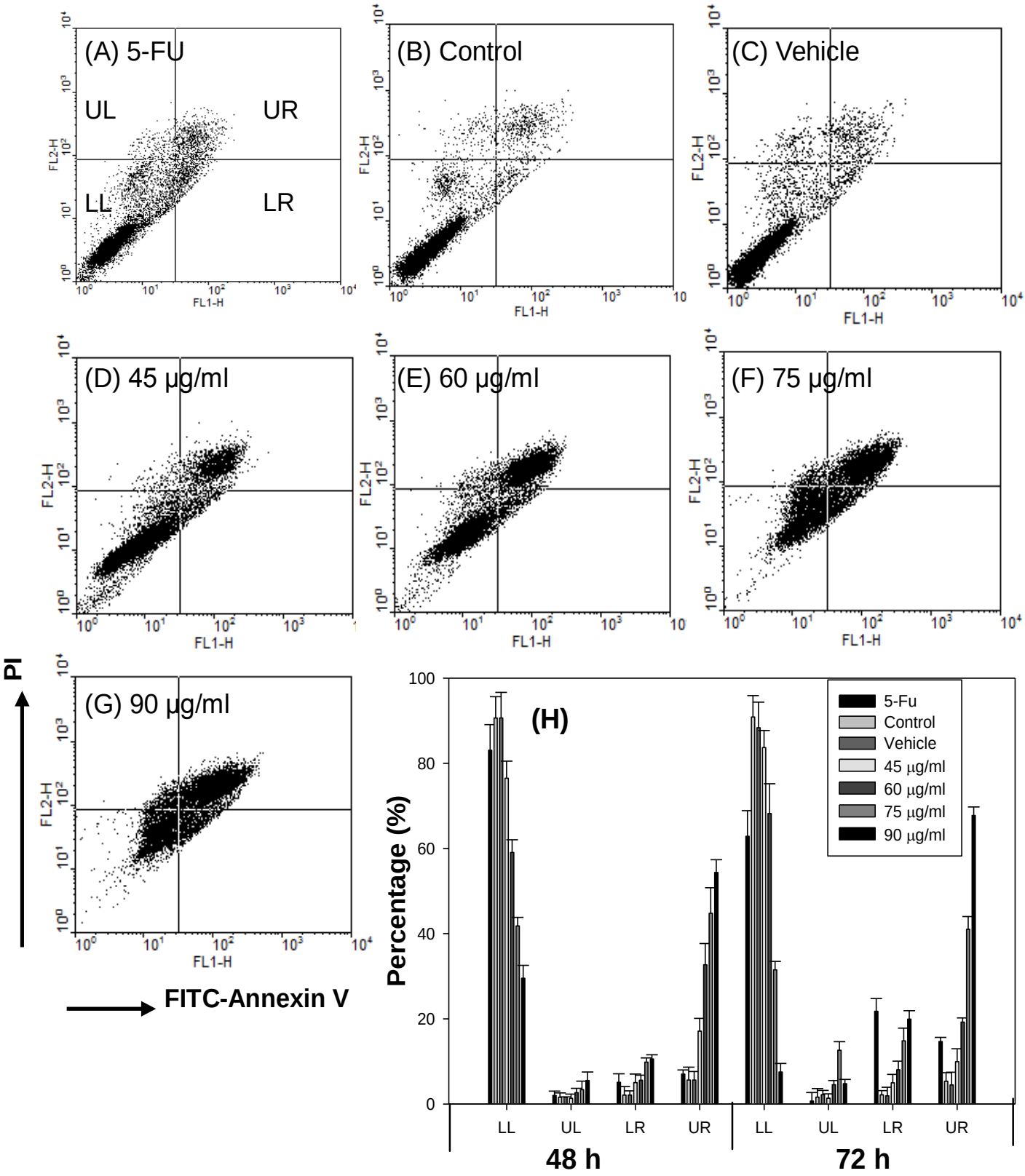

Figure 4. Effects of AIE on FaDu cell apoptosis. (A) Treated with 100 µg/ml 5-FU for 48 h, (B) untreated for 48 h, (C) treated with vehicle for 48 h, (D) treated with 45 µg/ml AIE for 48 h, (E) treated with 60 µg/ml AIE for 48 h, (F) treated with 75 µg/ml AIE for 48 h, (G) treated with 90 µg/ml AIE for 48 h, (H) percentage changes of the four quadrants under various treatments for 48 h and 72 h. Lower left (LL) quadrant: viable cells; lower right (LR) quadrant: early apoptotic cells; upper right (UR) quadrant: late apoptotic cells; upper left (UL) quadrant: necrotic cells.

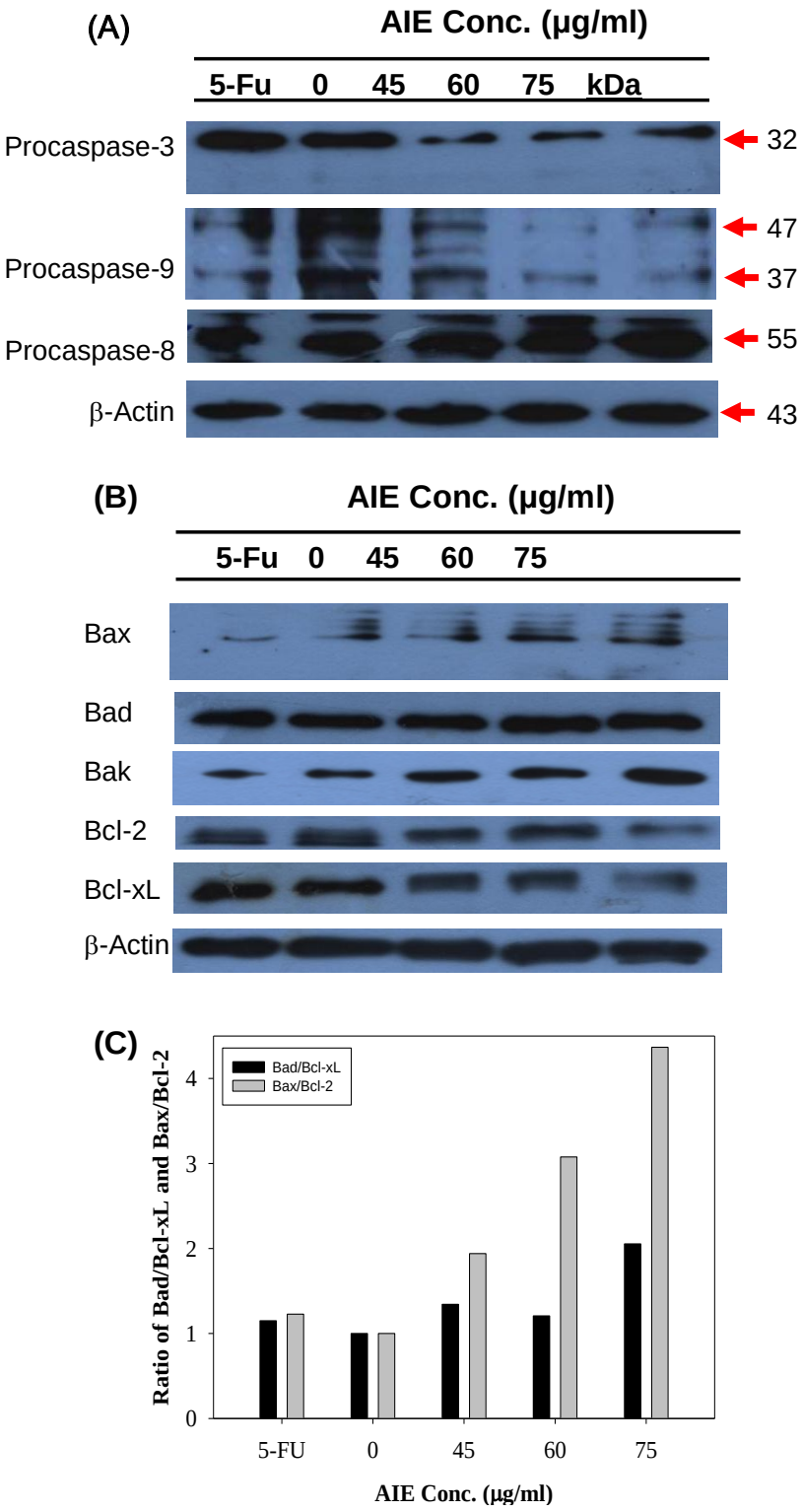

Figure 5. Effect of AIE on apoptosis-related proteins expression in FaDu cells. (A) Expression of procasepase-3, -9, and -8. (B) Expression of Bax, Bad, Bak, Bcl-2, and Bcl-xL. (C) The Bad/Bcl-xL and Bax/Bcl-2 ratios, which were analyzed by AlphaEaseFC software. FaDu cells of 1.5×10^6 cells were treated with 0 - 75 μg/ml AIE for 24 h. The cell extracts were subjected to electrophoresis on SDS-polyacrylamide gels with subsequent enzyme immunoassay using ECL. β-Actin was used as the internal control. Treatment of 100 μg/ml 5-FU for 24 h was used as a positive control.

1992; Webster et al., 1996). Recently, increasing attention has been paid to the use of plant materials for the complementary treatment of various cancers mainly because of the fact that natural agents may induce apoptosis of cancer cells, which is a useful mechanistic approach to cancer chemotherapy (Mohan et al., 2010a). The literature has shown extracts from *Agaricus brasiliensis* (Fan et al., 2011), *Gardenia jasminoides* (Lim et al., 2010), *Ginkgo biloba* (Kim et al., 2005), *Persea americana* (D'Ambrosio et al., 2011), *Rheum undulatum* L. (Choi et al., 2011) and *Terminalia catappa* L. (Yang et al., 2010). This investigation was aimed at examining the anti-proliferation effects of AIE on FaDu cells.

This study demonstrated that AIE has significant dose- and time-dependent anti-proliferative activity on FaDu cells *in vitro* (Figure 1). The normal lung fibroblast MRC-5 cells were also tested. From the MTT assay, the FaDu cells showed more sensitivity to AIE-induced growth inhibition (IC_{50} = 60.1 μg/ml) than did MRC-5 cells (IC_{50} = 102.7 μg/ml) under 24 h treatment, implying that AIE exhibited lower cytotoxicity against normal fibroblast cells than FaDu cells. Apoptosis is vital in maintaining cellular homeostasis, which is also a physiological process causing cellular self-destruction that involves specific morphological and biochemical changes in the nucleus and cytoplasm (Mohan et al., 2010b). Hence the therapeutic application of apoptosis is currently considered as a model for developing anti-cancer drugs (Schuchmann and Galle, 2004; Kundu et al., 2005). Apoptosis can be characterized by particular morphological changes, including plasma membrane bleb, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (Wyllie et al., 1980). Using phase-contrast inverse microscopy when FaDu cells were treated with AIE, this study observed typical morphological characteristics of apoptosis, such as cell shrinkage, rounding, apoptotic vacuoles, and forming majority of the floating cells (Figure 2).

Cell cycle control is a major event in cellular division. The mode of cell death induced by many natural compounds is closely associated with cell cycle arrest (Mohan et al., 2010b; Hsu et al., 2011). This study demonstrated that the cell cycle distribution of FaDu cells were altered following AIE treatment and was significantly arrested in the G2/M phase in a dose- and time-dependent mode. On the other hand, a concurrent reduction of cell population was observed in the G0/G1 phase (Figure 3). This finding reveals that AIE induced apoptosis on FaDu cells via G2/M cell cycle arrest. To investigate the probable mechanism of cell death, the presence of phosphatidylserine on the outer leaflet of apoptotic cells membrane was assessed using annexin-V-FITC/PI staining. This study elucidated that the higher the AIE concentration, the higher the cell number of late apoptotic dead cells (UR quadrant) rather than that of apoptotic cells (LR quadrant) (Figure 4). This study also observed that some cell dots shifted to the UL quadrant. Accordingly, AIE appeared to inhibit the proliferation of FaDu cells mainly via apoptosis and partly via necrosis.

Cell apoptosis can occur through two major pathways, including (1) the intrinsic or mitochondrial pathway and (2) the extrinsic or death receptor pathway (Yoon and Gores, 2002). The intrinsic pathway is triggered by the release of mitochondrial proteins, such as cytochrome c, which binds to apoptotic protease-activating factor 1 (Apaf-1), followed by the formation of Apaf-1-caspase-9 apoptosome complex and the activation of the initiator caspase-9 (Hengartner, 2000; Launay et al., 2005). Subsequently, caspase-9 induces procaspase-3 to become active caspase-3. Therefore, caspase-9 is essential in the intrinsic pathway of apoptosis (Johnson and Jarvis, 2004). On the other hand, the extrinsic pathway is initiated by the interaction of ligands with their respective death receptors, sequentially causing cleavage of initiator caspase-8. The active caspase-8 cleaves executioner caspase-3, resulting in apoptosis (Scaffidi et al., 1998). Therefore, caspase-8 is an important index for cell apoptosis through the extrinsic pathway. Furthermore, caspase-3 is considered an important downstream promoter in apoptosis, primarily because it can be activated by caspase-9 or -8 via intrinsic or extrinsic signaling pathways (Cohen, 1997; Zhang et al., 2004). This study illustrated that AIE induced the diminishing of procaspase-3 and procaspase-9, while the procaspase-8 expression of FaDu cells remained unchanged compared to untreated cells (Figure 5A). These results suggest that AIE can induce apoptosis in FaDu cells through the intrinsic pathway.

Moreover, Bcl-2 family proteins are crucial in the regulation of intrinsic apoptosis pathway (Mohan et al., 2010b). Anti-apoptotic Bcl-2 and Bcl-xL inhibit apoptosis by sequestering proforms of caspases or preventing the release of mitochondrial apoptogenic factors (Tsujimoto, 1998; Adams and Cory, 2007). Pro-apoptotic Bax, Bad and Bak inhibit Bcl-2 activity and promote apoptosis (Reed, 1995). This investigation showed that AIE treatments altered the expression of these proteins (Figure 5B). In addition, the elevation of the intracellular Bax/Bcl-2 ratio promoted the cell apoptosis (Figure 5C). Hence, the AIE-induced apoptosis was accompanied by the up-regulation of Bax and Bak and the down-regulation of Bcl-2 and Bcl-xL.

Conclusions

This investigation revealed that extract of *A. indica* had significant and direct cytotoxicity towards FaDu cells, and lower sensitivity on normal fibroblast MRC-5 cells. To our knowledge, this is the first study to demonstrate the probable mechanism of cancer cell death induced by this extract. The data of this study suggest that induced cell death in FaDu cells resulted mainly from the apoptotic effect and partly from the necrotic effect, and also in

association with cell cycle arrest. Furthermore, the apoptosis was through the intrinsic pathway and mediated by down-regulating Bcl-2 and Bcl-xL protein expression and up-regulating Bax and Bak expression. Further studies are required to evaluate the detailed molecular mechanisms and define the main bioactive phytochemicals in the extract.

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