Real-time Imaging of Apoptosis Induction of Human Breast Cancer Cells by the Traditional Chinese Medicinal Herb Tubeimu

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Abstract. *Traditional Chinese Medicine (TCM) has been used for thousands of years, including treatment for cancer. Use of modern technology and the scientific method to evaluate the efficacy of TCM for cancer should enable its more widespread use. In the present study, the efficacy of the TCM tubeimu, extracted from the tuber of the plant Bolbostemma paniculatum, on the MDA-MB-231 human breast cancer cell line was evaluated. The MDA-MB-231 cell line was engineered to express red fluorescent protein (RFP) in the cytoplasm and green fluorescent protein (GFP) linked to histone H2B in the nucleus, which allows real-time imaging of nuclear-cytoplasmic dynamics. Apoptosis was readily visualized in these cells by nuclear shape changes and fragmentation. The MDA-MB-231 RFP-GFP cells were cultured either in two-dimensions on plastic or in three-dimensions on Gelfoam®. Cells were treated with a dichloromethane extract of fresh tubeimu. Apoptosis was further monitored by DNA fragmentation determined by gel electrophoresis. Tubeimu induced apoptosis of MDA-MB-231 cells, as observed by fluorescence microscopy, as early as 24 hours of treatment in vitro in two-dimensional culture. By 48 hours' treatment, DNA fragmentation could be observed. The frequency of apoptosis increased through at least 72 hours' treatment, with most of the cells being killed. Tubeimu also induced apoptosis of MDA-MB-231 cells in three-dimensional culture on Gelfoam®, but to a lesser extent than in 2D culture. The results of the present study indicate the potential of tubeimu in breast cancer therapy.*

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The tuber (tubeimu) of *Bolbostemma paniculatum* (Maxim) Franquet (Cucurbitaceae), a traditional Chinese medicinal (TCM) plant, was listed in the Supplement to the Compendium of *Materia Medica,* written during the Qing Dynasty in 1765 (1).

Tubeimu has been shown to be active against nasopharyngeal carcinoma (2), cervical carcinoma (3), lung cancer (4), liver cancer (5), stomach cancer (6), colon cancer (7), and renal carcinoma (8). However, there have not been reports on the efficacy of tubeimu on breast cancer.

Our laboratory has developed a method for real-time imaging of apoptosis (9-11) by labeling cells with fluorescent proteins. To investigate the efficacy of tubeimu on breast cancer, we used the MDA-MB-231 human breast cancer cell line expressing red fluorescent protein (RFP) in the cytoplasm and green fluorescent protein (GFP) linked to histone H2B in the nucleus. Such dual-color cells are a useful tool for visualizing nuclear-cytoplasmic dynamics, including apoptosis, in real time, under fluorescence microscopy (9, 12).

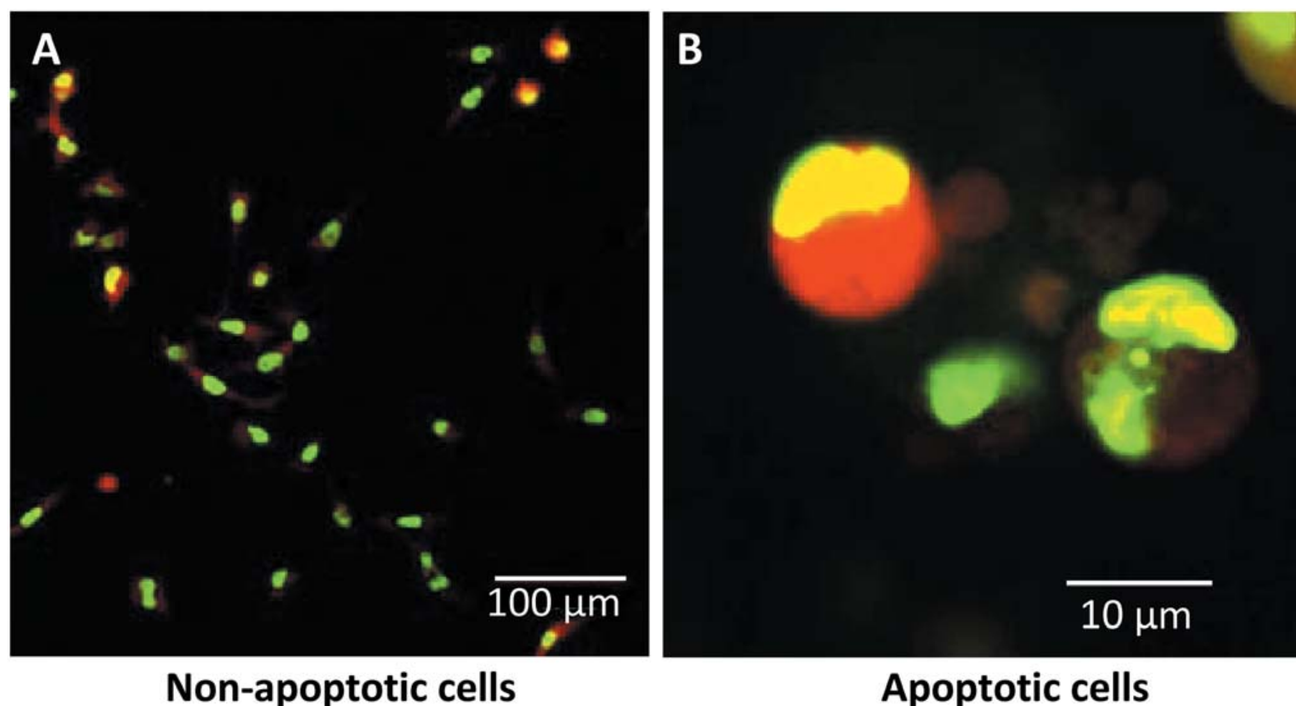

Figure 1. *Non-apoptotic and apoptotic MDA-MB-231 RFP-GFP cells. A: Non-apoptotic cells. B: Apoptotic cells.*

Efficacy of tubeimu was evaluated on MDA-MB-231-RFP-GFP human breast cancer cells in both 2D culture on plastic dishes and 3D culture on Gelfoam®, where cells take their natural shape and form 3D structures (11). Cancer cells and tumors cultured in a 3D gel-supported primary culture system, such as Gelfoam®, respond to drugs in an *in vivo*-like way (13).

Materials and Methods

Cell line. MDA-MB-231 RFP-GFP human breast cancer cells (AntiCancer Inc., San Diego, CA, USA) were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) at 37˚C with 5% CO_2.

2D Culture and drug testing. Fresh tubeimu was extracted with dichloromethane and dissolved in phosphate-buffered saline (PBS) (for stock and working solutions), and then mixed with RPMI-1640 at concentrations of 15 µg/ml, 20 µg/ml, and 40 µg/ml. A total of 10^6 MDA-MB-231 RFP-GFP cells were seeded on 35 mm plastic dishes for 2D culture or on Gelfoam® (Pharmacia & Upjohn Co., Kalamazoo, MI, USA) for 3D culture, for tubeimu testing (please see below). Treatment was either for 24, 48 or 72 h. Untreated cultures served as controls. Each treatment or control was tested in triplicate.

Three-dimensional Gelfoam® culture and drug testing. Gelfoam® was cut in 10×10×3 mm pieces and soaked in RPMI-1640 medium. 10^6 MDA-MB-231-RFP-GFP cells were seeded on hydrated Gelfoam®, in RPMI-1640 medium with 10% FBS, with sufficient volume to cover the Gelfoam® in 35 mm dishes. Care was taken so that cells did not float away from the Gelfoam® and scatter in the medium. Tubeimu was prepared, as above, at 20 µg/ml and 25 µg/ml, for testing in Gelfoam® 3D culture.

Confocal imaging. A Fluorview FV1000 fluorescence confocal microscope (Olympus, Tokyo, Japan) was used (14). Images of MDA-MB-231 RFP-GFP cells (Figure 1) were acquired at 24, 48, and 72 h of treatment with tubeimu in 2D or 3D culture.

DNA gel electrophoresis. Treated and control MDA-MB-231 GFP-RFP cells (0.5-1×10^6) were placed in 1.5 ml microcentrifuge tubes and centrifuged for 5 min at 500 ×*g*. The supernatant was removed with a pipette. The pelleted cells were then lysed in 35 µl Tris EDTA (TE) lysis buffer by gentle pipetting. Care was taken not to shear the DNA. RNAse A solution (5 µl) was added to the lysate mix and the tubes were incubated at 37˚C for 5-10 min. Proteinase K solution (5 µl) was added to each tube and mixed and the tubes were then incubated at 50˚C for 30 min or longer until the lysates became clear. Ammonium acetate solution (5 µl) was added, the solution was mixed and kept at –20˚C for at least 10 min. The sample was then centrifuged for 10 minutes (16000 ×*g*) to precipitate the DNA. The supernatant was removed and the DNA pellet was washed with 0.5 ml of ice-cold 70% ethanol. The DNA pellet was then air dried for 10 minutes at room temperature. The dried DNA pellet was dissolved in 30 µl DNA suspension buffer and then the sample (20 µl) was loaded onto a 1% Tris-acetate agarose gel containing 0.5 µg/ml ethidium bromide in both the gel and the running buffer. The gel was electrophoresed at 5 V/cm for 1-2 h, stained in TE/ethidium bromide for 10-15 min, and visualized by transillumination under UV light and photographed.

Statistical analysis. The Student's *t*-test was used to obtain *p*-values in order to compare tubeimu-treated and untreated cultures.

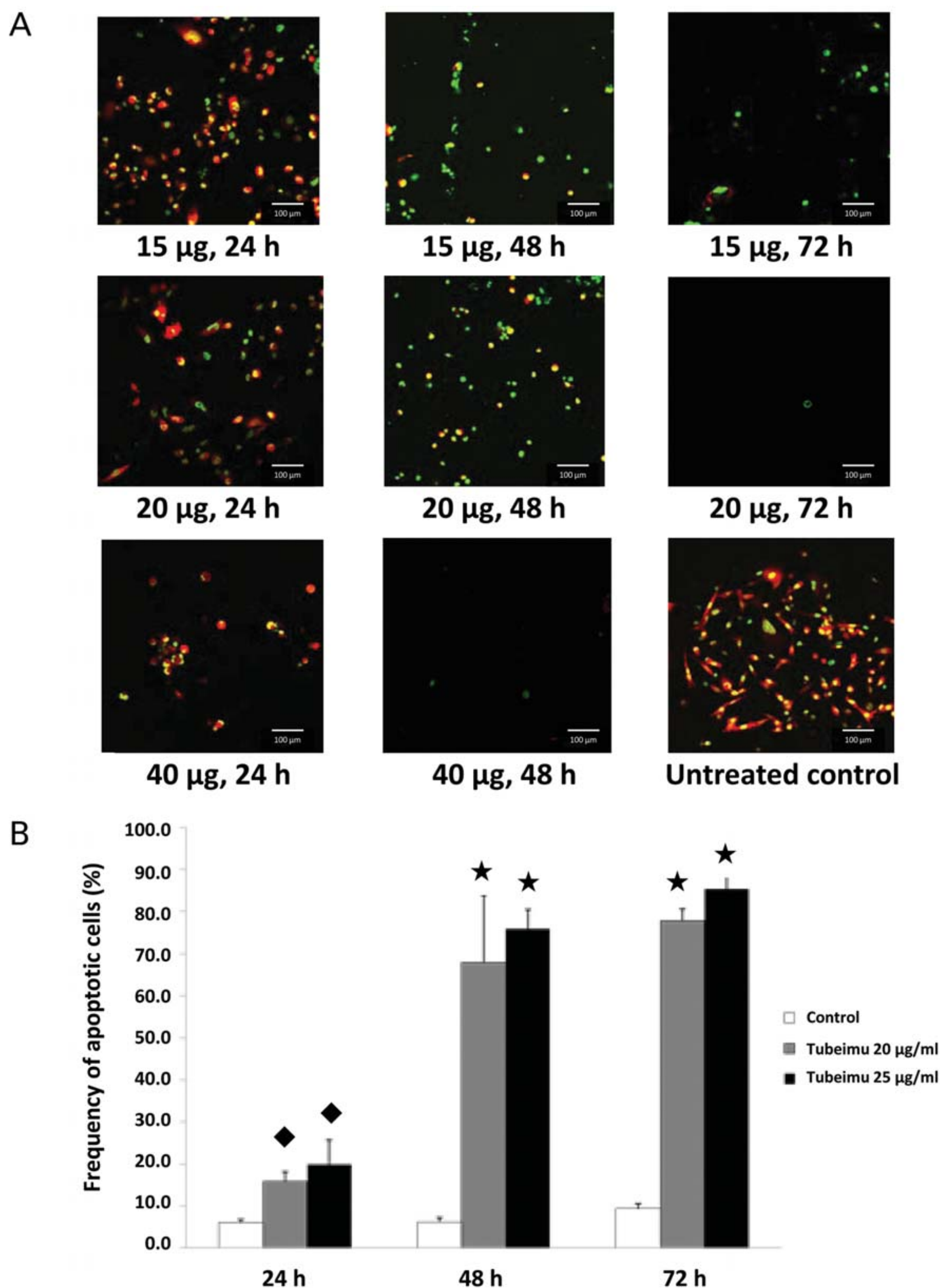

Figure 2. *A, B. Apoptosis and killing of MDA-MB-231-RFP-GFP cells by tubeimu in 2D culture. MDA-MB-231 cells were cultured on plastic dishes in RPMI-1640 medium. The induction of apoptosis by tubeimu in MDA-MB-231 RFP-GFP cells in 2D culture was dose- and time-dependent as observed after treatment with 15 µg/ml, 20 µg/ml, and 40 µg/ml tubeimu for 24, 48, and 72 hours. The decrease in cell number was also dose- and time-dependent compared to untreated control ($p<0.01$). Bar=100 µm. (◆=$p<0.05$, ★=$p<0.01$).*

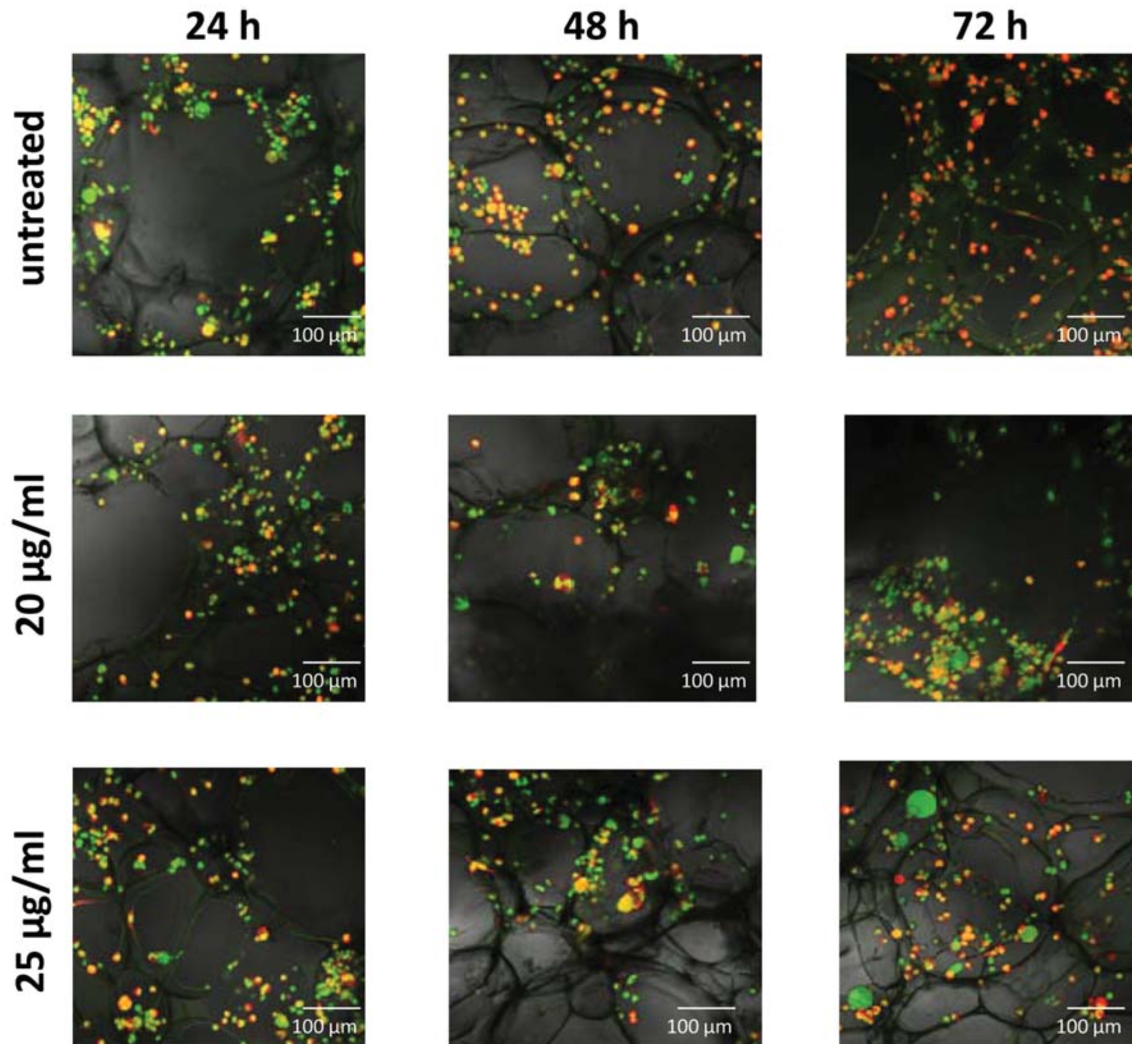

Figure 3. *Apoptosis and killing of MDA-MB-231 cells by tubeimu in 3D culture. MDA-MB-231 cells were cultured on Gelfoam® in RPMI-1640 medium and treated with 20 µg/ml and 25 µg/ml tubeimu for 24, 48 and 72 hours. Bar=100 µm.*

Results and Discussion

Induction of apoptosis of MDA-MA-231 RFP-GFP human breast cancer cells by tubeimu in 2D culture. Apoptosis was observed in the tubeimu-treated MDA-MD-231 RFP-GFP cells growing on plastic dishes in 2D culture by nuclear size changes and progressive nuclear fragmentation using confocal imaging (Figure 1). The MDA-MB-231 RFP-GFP cells displayed obvious apoptotic morphological changes after being treated with tubeimu for 24 h. Nuclear size changes, chromatin condensation and progressive nuclear fragmentation were observed (Figure 1). The induction of apoptosis by tubeimu in MDA-MB-231 RFP-GFP cells in 2D culture was dose- and time-dependent, as observed after treatment with 15 µg/ml, 20 µg/ml, and 40 µg/ml tubeimu for 24, 48, and 72 h (Figure 2A, B). The decrease in cell number was also dose- and time-dependent (p<0.01) (Figure 2A). Tubeimu treatment at 40 µg/ml resulted in irregular morphology of the cells and the cell number decreased sharply (Figure 2A). Almost no live cells remained after 48 h treatment with 40 µg/ml tubeimu (Figure 2A). By 72 h, there were almost no live cells remaining even after treatment with 20 µg/ml tubeimu (Figure 2A).

Induction of apoptosis of MDA-MB-231 RFP-GFP human breast cancer cells by tubemu in 3D culture. In 3D Gelfoam® culture, the MDA-MB-231 RFP-GFP cells were more resistant to tubeimu when tested at 20 µg/ml and 25 µg/ml for 24, 48, and 72 h (Figure 3). In 2D culture, the difference between the three different tubeimu dosages and

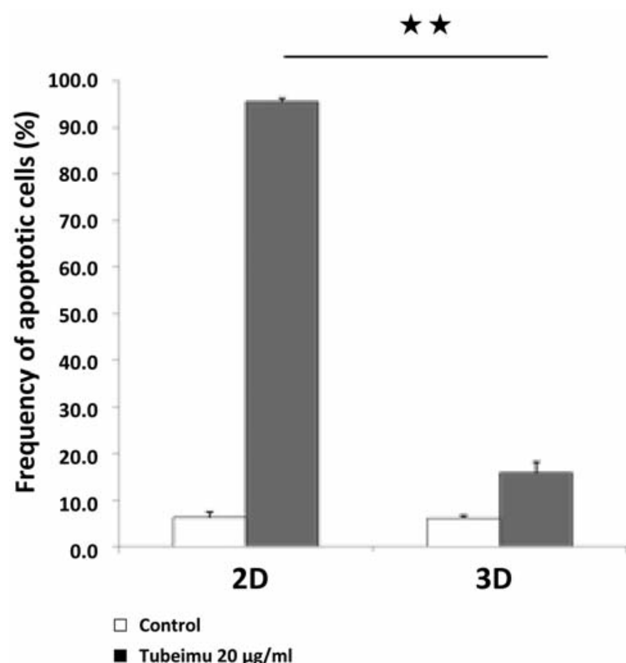

Figure 4. *Comparison of apoptosis in MDA-MB-231 RFP-GFP cells induced by tubeimu in 2D and 3D cultures. MDA-MB-231 RFP-GFP cells were cultured in 2D on plastic dishes and 3D on Gelfoam® in RPMI-1640 medium. Please see Figures 2 and 3 for details. The frequency of apoptosis in treated and control cultures was compared (★★=p<0.01).*

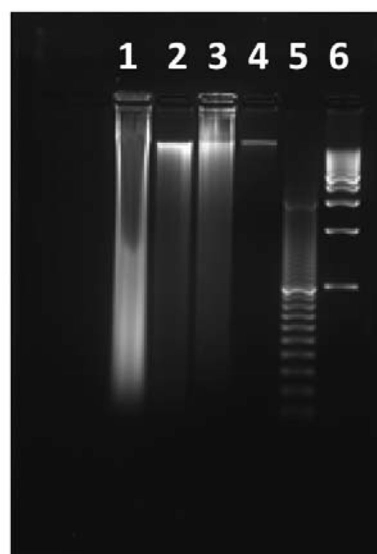

Figure 5. *Fragmentation of MDA-MB-231 RFP-GFP cells after tubeimu treatment. Lanes contain DNA from the following cultures: (1) tubeimu at 15 μg/ml for 72 hours in 2D culture, (2) tubeimu at 15 μg/ml for 48 hours, (3) tubeimu at 15 μg/ml for 24 hours, (4) untreated control, (5) DNA marker (100bp), (6) DNA marker (smallest size is 1,000 bp).*

the three different timepoints were more obvious than in 3D culture (Figures 2A, 3 and 4). In 3D Gelfoam® culture, cancer cells assume their natural shape (more *in vivo*-like) (15). In addition, the cells in 3D Gelfoam® culture attach more tightly to each other and have a broader contact area with each other than that in 2D culture, which may protect the cells from entering apoptosis. However, in the control group in 3D culture at all time points, the cell number increased, in contrast with the number of tubeimu-treated cells which decreased in number over this time period (p<0.01) (Figures 3 and 4). In 3D Gelfoam® culture, the cell morphology changed in the treated cells compared with that of the control group (Figure 3).

Induction of DNA fragmentation in MDA-MD-231 RFP-GFP cells by tubeimu. Agarose-gel electrophoresis detected DNA fragmentation in the MDA-MB-231 RFP-GFP cells after 48 h treatment with tubeimu in 2D culture, with an increase at 72 h. In contrast, morphological changes observed by fluorescence microscopy revealed apoptosis induction by tubeimu by 24 h. Fluoresence microscopy of cancer cells labeled with GFP in the nucleus and RFP in the cytoplasm is therefore more sensitive than DNA electrophoresis for detecting apoptosis (Figure 5).

TCM is a vast potential resource for cancer treatment. However, in order to determine which herbs or their combination have anticancer activity that can be used widely, modern methods must be used to evaluate them. In the present report, we utilized human breast cancer cells engineered to express RFP in the cytoplasm and GFP in the nucleus (9-11). These cells clearly report nuclear-cytoplamic dynamics in real time and are ideal to reveal the onset and progression of treatment-induced apoptosis. The findings of this report, demonstrate the potential of tubeimu for cancer therapy.

Conflict of Interest

None of the Authors have a conflict of interest with this study.

Acknowledgements

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