ฤทธิ์ความเป็นพิษต่อ glioma เซลล์ (ASK) และอะโพโทซิสของสารสกัดจากฟ้าทะลายโจร
Cytotoxicity and Apoptosis effect of Crude Extract of *Andrographis paniculata* Nees. (APE) on rat glioma cells (ASK)

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บทคัดย่อ

ฟ้าทะลายโจรเป็นสมุนไพรที่มีฤทธิ์ทางเภสัชวิทยากว้าง และถูกใช้ในทวีปเอเชียมาหลายศตวรรษ ฟ้าทะลายโจรมีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งหลายชนิด แต่ฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งไม่เคยทดสอบใน glioma การศึกษานี้ทดสอบฤทธิ์สารสกัดฟ้าทะลายโจรต่อการยับยั้งการเจริญของ glioma จากหนู (ASK) โดยศึกษาความเป็นพิษต่อเซลล์และผลต่อการเกิดอะโพโตซีส โดยศึกษารูปร่างของเซลล์และดีเอนเอ การศึกษานี้พบว่าสารสกัดฟ้าทะลายโจรทำให้เซลล์ตายเป็นสัดส่วนเพิ่มขึ้นตามความเข้มข้นของสารสกัดฟ้าทะลายโจรที่เพิ่มขึ้น โดยเซลล์ที่ตายไม่เกาะติดกับผิวภาชนะที่เลี้ยง ทำให้มีรูปร่างกลม แทนที่จะเกาะพื้นเป็นรูปกระสวย สารสกัดที่ความเข้ม 110 ± 4.5 µg/ml ยับยั้งการเจริญของ glioma ได้ 50% การย้อมสีนิวเคลียสด้วย DAPI และ Propidium iodide พบว่าเมื่อเลี้ยงเซลล์ด้วยสารสกัดฟ้าทะลายโจร 110 µg/ml นาน 48 ชั่วโมง พบเซลล์ที่มีลักษณะปกติ 5.32 ± 0.97% และเซลล์ที่มีลักษณะของอะโพโตซีสระยะแรก 86.07 ± 5.5% และเซลล์ตายแบบอะโพโตซีสระยะหลัง 8.61 ± 1.25% นอกจากนี้พบสายสั้นของสายพันธุกรรมซึ่งแสดงถึงอะโพโตซีสเพิ่มขึ้นตามความเข้มข้นของสารสกัดฟ้าทะลายโจรที่เพิ่มขึ้น

การศึกษานี้พบว่าสารสกัดฟ้าทะลายโจรสามารถทำให้เซลล์ตาย และการเปลี่ยนแปลงรูปร่างของเซลล์ที่เป็นลักษณะของอะโพโตซีสเช่น ผนังเซลล์เป็นตุ่ม, โครมาตินหนาแน่น, นิวเคลียสและสายพันธุกรรมเป็นสายสั้น ๆ ดังนั้นสารสกัดฟ้าทะลายโจรสามารถยับยั้งการเจริญของ glioma ได้

คำสำคัญ : ฟ้าทะลายโจร, เซลล์มะเร็ง ASK, ยับยั้งการเจริญเติบโต, อะโพโตซีส, ดี เอ็น เอ แตก

Abstract

Andrographis paniculata Nees. has a wide spectrum of pharmacological activities and has been used for centuries in Asia. Recently, anticancer activity of *Andrographis paniculata* extract (APE) has been tested on different types of human cancers cell lines. However, the anti-cancer effect of APE in glioma has not been determined. In this study, we examined whether the APE inhibit rat glioma cells (ASK) and characterized apoptosis in both morphological and biochemical features.

APE-induced cell death was associated with round cells, lost of cell-to-cell contact and fewer adherent cells when compared with cuboid and polygonal in normal shape. The IC_{50} values for APE was 110 ± 4.5 µg/ml. Apoptotic nuclei were quantified using fluorescence double staining: DAPI and propidium iodide. APE-treated cells exhibited chromatin condensation, and nuclear fragmentation as compared to control. Quantitative estimation of apoptotic nuclei in APE-treated cells (110 µg/ml for 48 h) was 5.32 ± 0.97% (normal cell), 86.07 ± 5.5% (viable cells with apoptotic nuclei) and 8.61 ± 1.25% (necrosis or late apoptotic nuclei). The oligonuclesomal DNA fragmentation in agarose gel was observed in a dose-dependent manner when cells were treated with 50, 110 and 160 µg/ml APE for 48 h.

These results indicated that APE-induced cell death via morphological changes typical of apoptosis including membrane blebbing, chromatin condensation, nuclear and DNA fragmentation. The induction of apoptosis by APE could be considered as potential sources of anti-cancer compounds in glioma.

Keywords: *Andrographis paniculata* Nees., ASK cells, Antiproliferation, Apoptosis, DNA fragmentation

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Introduction

Andrographis paniculata Nees. is in family *Acanthaceae*, and has been used for centuries in Asia to treat common cold, tonsillitis as an anti-inflammation and immunostimulant. It was also found in Indian herbal medicine, Ayurvedic formulas (Dahanukar *et al.*, 2000). In traditional Chinese medicine, it has bitter tonic properties and used to decrease the body heat and to increase toxins secretion from body (Tang and Eisenbrand, 1992). In Scandinavian countries, it is commonly used to prevent and treat common colds (Coon and Ernst, 2004). It has been also selected for herbal medicines to prevent or treat of uncomplicated common cold in Thailand (Sawasdimongkol *et al.*, 1990). Extensive research revealed that this herbal extract has a wide spectrum of pharmacological activities, including anti-diarrhea (Gupta *et al.*, 1990), anti-inflammatory (Hidalgo *et al.*, 2005), antibacterial (Ahmad *et al.*, 1998), anti-HIV (Calabrese *et al.*, 2000), immuno-stimulant (Puri *et al.*, 1993; Kumar *et al.*, 2004), hepatoprotective (Visen *et al.*, 1993; Kapil *et al.*, 1993; Trivedi *et al.*, 2000), antiplatelet aggregation (Thisoda *et al.*, 2006) anticancer (Cheung *et al.*, 2005; Zhou *et al.*, 2006).

Recently, anticancer effect of *Andrographis paniculata* extract (APE) has been reported on different types of human cancer cell lines. Interestingly, methanol extract of APE and its isolated compounds (14 - deoxy andrographolide, neoandrographolide) had growth inhibitory on mouse myeloid leukaemia (M1) cells (Matsuda *et al.*, 1994). Ethanol extract of APE and diterpenoid components of APE inhibited human acute myeloid leukemic HL-60 cells by cell-cycle arrest at G0/G1 phase and mitochondrial-mediated apoptosis (Cheung *et al.*, 2005). Andrographolide, the major diterpenoid of the *Andrographis paniculata*, induced apoptosis in human cervical (HeLa), hepatoma (HepG2) and breast (MDA-MB-231) cancer cell line via extracellular and intracellular apoptosis (Zhou *et al.*, 2006). In addition, Andrographolide had direct anticancer effect on cancer cells via cell-cycle arrest at G0/G1 phase through induction of cell-cycle inhibitory protein p27 and decreased cyclin-dependent kinase 4 (CDK4) expressions (Rajagopal *et al.*, 2003). According to these evidences, it may be an effective, non-invasive strategy for suppress tumor development.

Glioma is a glial cell brain tumor. The high-grade gliomas are undifferentiated, high-vascular tumors which had high tendency to invade to normal brain (Wong *et al.*, 1999). Standard therapy for glioma is a combine of surgery, and chemotherapy. One of the major problems in treating cancer is a tendency of glioma to produce a second primary tumor. There are ongoing research in the topics of treatment of glioma include apoptosis, stop blood vessels of tumors and efficiency of combinations of different treatments (Wong *et al.*, 2007). The development of herbal medicine that has a toxic effect to glioma but has little or non toxic to normal cells remains one of the most challenging areas in cancer research.

The effect of APE as anti-cancer agents in glioblastoma has not been determined. In this study, we examine the inhibitory effect of APE on ASK cell and to characterize the morphological and biochemical features of apoptosis.

Materials and methods

Chemicals

The following chemicals were purchased from the following suppliers: propidium iodide (PI), 4'-6-Diamidino-2-phenylindole (DAPI) and SYBER Gold from invitrogen, Ltd. (Paisley, UK); dimethyl sulfoxide (DMSO) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT) from Sigma Chemical Co. (St Louis, MO, USA); Cell culture media or materials were purchased from Gibco BRL (Gaithersburg, MD, USA) and InVitromex (Grevenbroich, Germany).

Plant material and preparation of extract

The stems and leaves of *Andrographis paniculata* Nees. (Acanthaceae) were harvested in March 2007 from Dongbang District, Prajeanburi, Thailand. The stems and leaves were air dried. The 46.82 grams of dried plant were extracted with ethyl acetate at room temperature. The extracts were concentrated by rotary evaporator to give 0.3889 grams of crude extract. The crude extract of APE was dissolved in dimethylsulfoxide (DMSO) to obtain 1 mg/ml stock solution and then filtered through a 0.22 μm cellulose nitrate membrane and stored at -20 °C before use. Dilution was dissolved in phosphate buffer saline (PBS). A voucher specimen No.5002 was deposited in Department of chemistry, Burapha University.

Cell culture and MTT assay

ASK cell lines, rat glioma cells, were obtained from National Cancer Institute of Thailand. Cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 1mM sodium pyruvate, and 100 U/ml penicillin, and 100 μg/ml streptomycin and kept at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air mixture.

ASK cells in logarithmic growth phase were collected. After digestion with trypsin-EDTA, 2×10^5 cells/well were plated in flat-bottomed 96-well plates in 100-ml volumes and incubated for 24 h at 37 °C under a humidified 5% CO_2 and 95% air mixture to allow cell attachment. Cells were treated with 0.3% DMSO (vehicle control), Doxorubicin 0-10 μg/ml (positive control) and APE 30-250 μg/ml (test sample) for 48 h under the same conditions. At the end of 48 h, 20 μl of 0.5% MTT in PBS were added to each well and plates were incubated at 37 °C for 3 h. At the end of 3 h, the supernatant was removed and replaced with 100 μl of DMSO. The metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells into an insoluble colored formazan product was measured with a microplate spectrophotometer reader (Cecil Bioquest 2000 Series) at 570 nm. At least three separate experiments for each sample were used to determine the cell viability. Under these conditions, 0.3% DMSO was not toxic and cell survival in vehicle control was assumed 100%. The percentage of cell viability was calculated on the basis of the following formula:

$$\text{\% Cell viability} = \frac{\text{Absorbance at 570 nm of treated cells}}{\text{Absorbance at 570 nm of control cells}} \times 100$$

Fluoresent microscopic analysis of nuclear fragment

Glioma at concentration 5×10^4 cells/ml were cultured in polystylene flask and incubated for 24 h at 37 °C, 5% CO_2 and 95% air mixture. Then, APE 110 μg/ml was added and further incubated for 48 h in the same condition. Doxorubicin 1.7 μg/ml was also added into another flask as a positive control. Cell grown in the presence of 0.3% DMSO was used as a negative control. The morphology of the cells nuclei was quantified after fluorescence dyes staining. Briefly, at designated time points, cells were collected and washed in PBS. The cells were then incubated with RNase A (20 mg/ml, 5 μl) in the dark for 30 minutes at room temperature. Harvested cells were washed three times with PBS, and then resuspended in PBS containing 5 μg/ml DAPI for identifying nuclear fragmentation, and 5 μg/ml propidium iodide, for identifying non-viable cells, for 30 min at 37 °C. The supernatant were discarded to remove unbound dye. The 10 μl of cells were mounted on the slide, covered with a coverslip and sealed the edges with nail polish. Condensed or fragmented nuclei in viable cells were then visualized through blue and green filter of fluorescence microscope (Olympus BX51) at 400 magnification. Normal nuclei can be identified by glowing bright and homogeny while apoptotic nuclei are condensed chromatin and fragmented morphology of nuclear bodies. For each treatment group, 200-400 different nuclei were counted in random microscopic fields. Data were expressed as percentage of nuclei in different phases. At least three separate experiments for each sample were performed.

DNA extraction and electrophoresis analysis

The GF-1 Tissue DNA Extraction Kit (Vivantis) was used according to the manufacturerӨs instructions. After treatments, floating and adherent cells were washed with PBS and then lysed with digestion buffer containing proteinase K (20 mg/ml, 20 μl) at 60°C overnight. RNaseA (20 mg/ml, 10 μl) was added and incubated for 5 min at 37°C. Genomic DNA was extracted with ice-cold absolute ethanol. DNA (400 ng) were mixed with SYBER Gold (0.1 mg/ml, 1 μl) and loading buffer and loaded onto pre-solidified 1.5% agarose. The agarose gels were run at 150 V for 60 min in TBE buffer. Gels were observed and photographed under transilluminator (Clare Chemical Research).

Data processing

Data were expressed as mean ± standard error of the mean (S.E.M) from independent 3-4 experiments and analyzed with the software Microcal™ Origin 6.

Results and Discussion

ASK is an excellent model of a tumor of the brain that arised from glial cells and were similar to those found in human gliomas (Singh *et al*., 2003). ASK cells were treated with 30-250 μg/ml of APE for 48 h and cellular viability was analyzed by MTT assay. The APE markedly decreased viable cell numbers in a dose-dependent manner (Fig 1). At 200 μg/ml of APE, the viability of glioma cells was reduced to less than 10%. The APE was less toxic than Doxorubicin in that the cell number decreased to less than 10% at concentrations 8 μg/ml. The IC_{50} values for APE and Doxorubicin on ASK cells was 110 ± 4.5 μg/ml and 1.7 ± 0.07 μg/ml, respectively. From this result, the IC_{50} value was selected for subsequent experiments. Morphological changes could be seen after 48 h treatment with both APE and Doxorubicin which was characterized by round cells, lost of cell-to-cell contract and fewer adherent cells when compared with cuboid and polygonal in normal shape. This result was in agreement with the finding of other studies, which found that APE and its components can inhibit the growth of human colon cancer (HT-29) cells and HL-60 cells. The IC_{50} of methanol APE extract was 10 μg/ml and its fractions ranged from 10-46 μg/ml in HT-29 cells (Kumar *et al*., 2004). In HL-60 cells, The IC_{50} of ethanol extract was 14.01 μg/ml while it was less effective in other cancer cells (Cheung *et al*., 2005).

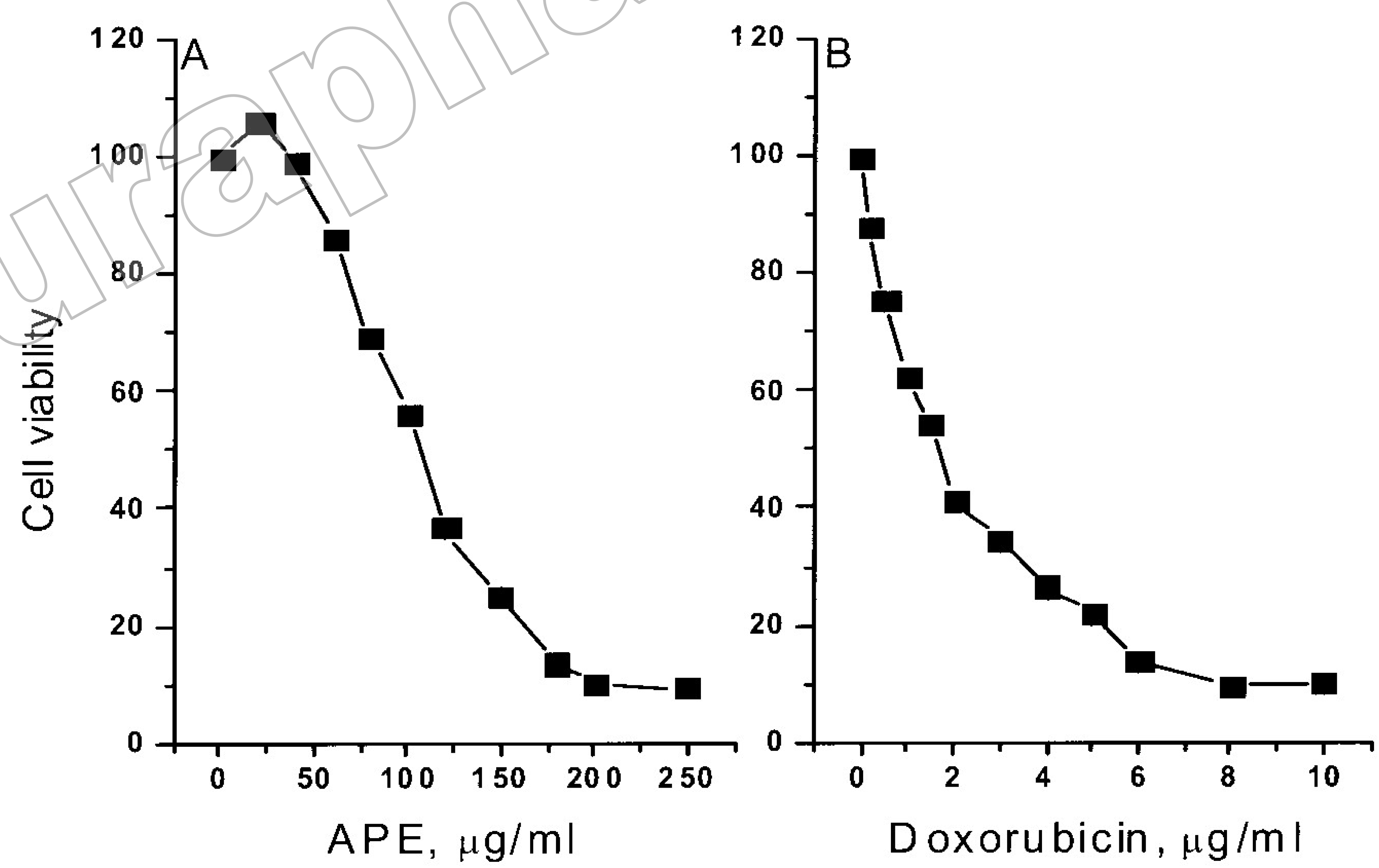

Fig. 1 Antiproliferative effects of APE (A) and Doxorubicin (B) to ASK cells. Cells (210^5 cells/well) were exposed to APE (30-250 μg/ml) or Doxorubicin (0 - 10 μg/ml) for 48 h. Viable cell number was measured with MTT assay. Data were expressed as mean ± S.E.M of 4 replicates.

Table 1. Percentage of nuclei staining with DAPI and PI. ASK cells were treated with APE (110 μg/ml) and Doxorubicin (1.7 μg/ml) for 48 h. The percentage of cells with a normal or condensed or fragmented nucleus was estimated by counted directly with fluorescence microscope. The values are mean ± S.E.M of 2-3 replicates.

	% Normal cells (Homogenous DAPI staining)	**% Apoptosis cells** (Condensed or fragmented DAPI staining)	**% Late apoptosis or necrotic cells** (PI staining)
0.3% DMSO	93.9 ± 13.66	2.53 ± 0.33	3.55 ± 1.18
APE 110 μg/ml	5.32 ± 0.97	86.07 ± 5.5	8.61 ± 1.25
Doxorubicin 1.7 μg/ml	2.81 ± 0.86	42.25 ± 3.37	54.92 ± 5.15

Whether APE is involved in apoptosis of ASK cells, morphology of apoptosis and the DNA laddering were investigated. Both DAPI and PI staining are fluorescent nuclear dyes. DAPI can pass through an intact cell membrane but PI is membrane impermeant that commonly used for identifying necrosis or late apoptotic cells. ASK cells treated with 0.3% DMSO had rounded nuclei with homogenous DAPI staining and defined plasma membrane contour, but ASK cells treated with 110 μg/ml APE had plasma membrane blebbing, condensation of chromatin, nuclear fragmentation and formation of apoptotic bodies (Fig 2). In control group, the quantitative estimation of normal cells was 93.9 ± 13.66%, the viable cells with apoptotic nuclei was 2.53 ± 0.33% and the necrosis or late apoptotic nuclei was 3.55 ± 1.18%. When the ASK cells were treated with 110 μg/ml APE for 48 h, the quantitative estimation of normal cells was 5.32 ± 0.97%, the viable cells with apoptotic nuclei was 86.07 ± 5.5% and the necrosis or late apoptotic nuclei was 8.61 ± 1.25%. In the treatment with 1.7 μg/ml Doxorubicin for 48 h, the quantitative estimation of normal cells was 2.81 ± 0.86%, the viable cells with apoptotic nuclei was 42.25 ± 3.37% and the necrosis or late apoptotic nuclei was 54.92 ± 5.15%.

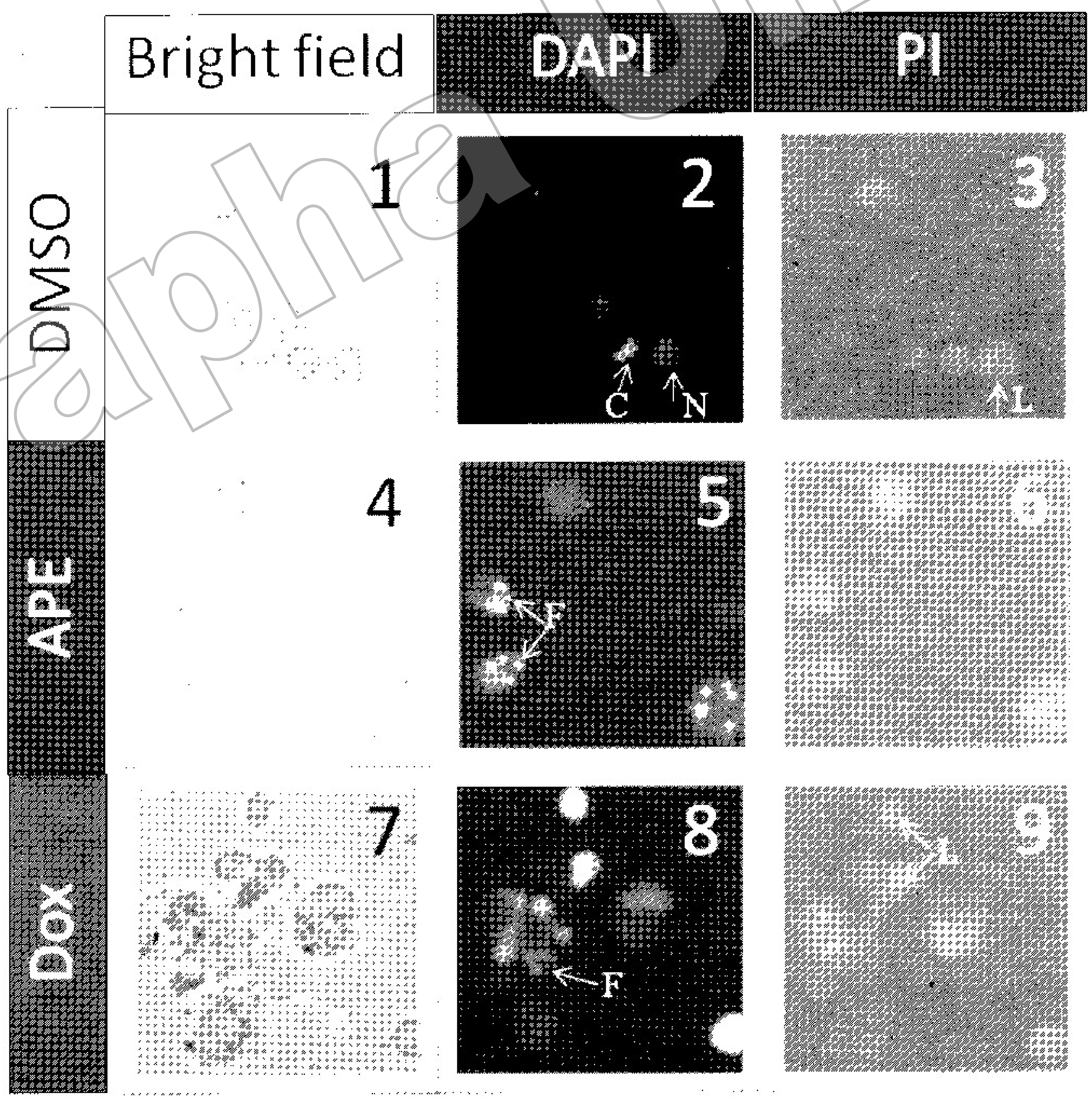

Fig. 2 Morphological features of nuclei of ASK cells treated with 0.3% DMSO (1-3), APE 110 μg/ml (4-6) and Doxorubicin (Dox) 1.7 μg/ml (7-9) for 48 h. Cells were observed by bright field morphology (1, 4, 7), DAPI staining (2, 5, 8) and PI staining (3, 6, 9). N: normal nuclei, C: chromatin condensation, F: nuclear fragmentation, L: late apoptosis and B: membrane blebbing.

Doxorubicin mediated cell death by stimulating apoptotic pathway through irreversible DNA damage with subsequent mitotic failure (Bold *et al.*, 1997). Drastic decrease in viable apoptotic cells together with increase in late apoptotic cells was noticed in Doxorubicin-treated cells. Since Doxorubicin is more toxic than APE, it might also be that the apoptotic cells under the in vitro cell culture conditions cannot undergo rapid phagocytosis as in vivo in the intact tissue (Grub *et al.*, 2000). DNA laddering, another method investigated for apoptosis, was carried out by agarose gel electrophoresis. DNA fragmentations were observed when cells were treated with 50, 110 and 160 µg/ml of APE for 48 h (Fig 3). APE-induced apoptosis of ASK cells showed a progressive increase in nonrandom fragmentation into a ladder with a concentration-dependent manner. The caspase activity in ASK cells treated with APE is not investigated in this study.

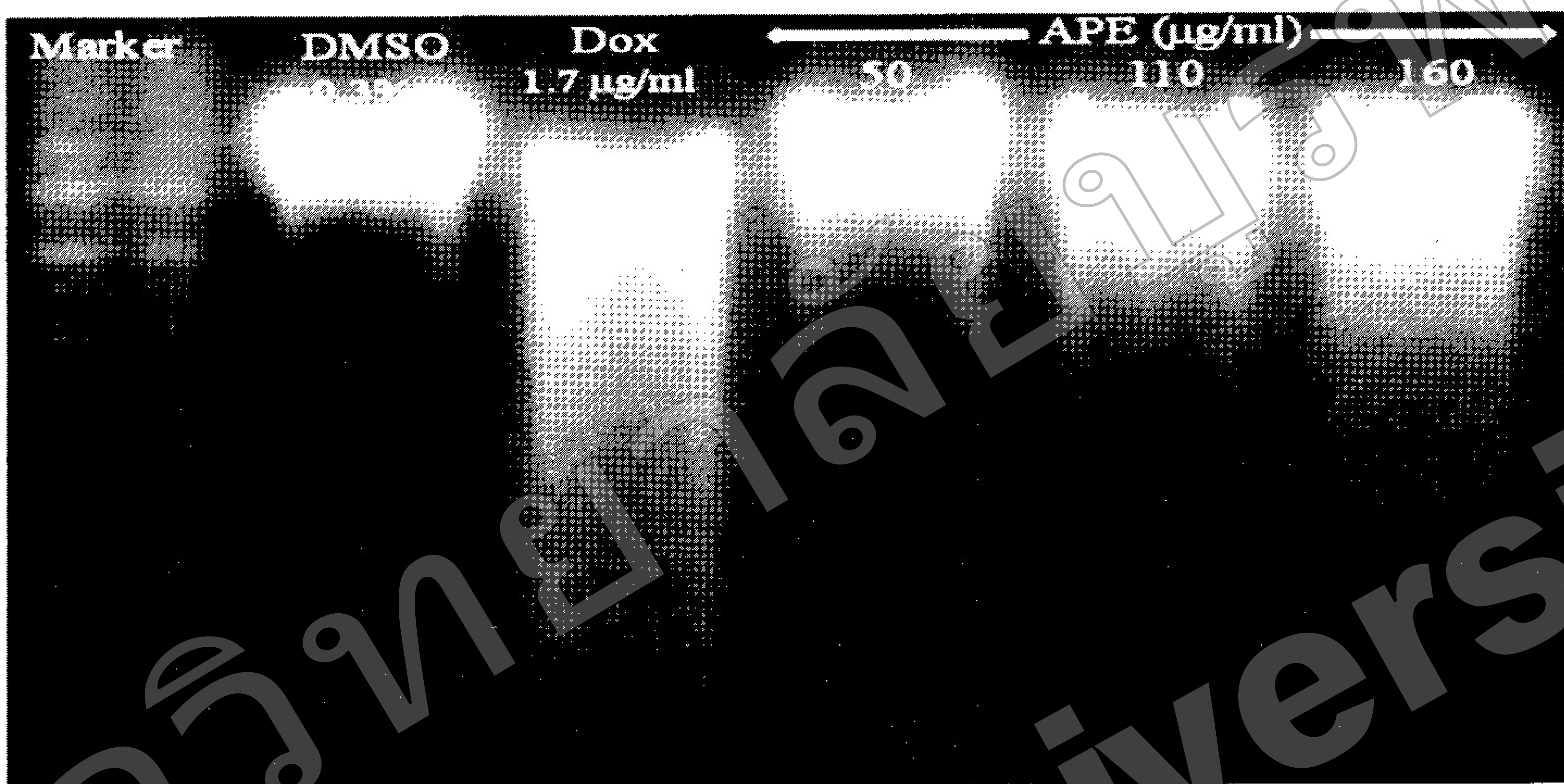

Fig. 3 A photograph of the SYBER Gold -stained gel, which is representative of three independent experiments, is shown. DNA fragmentation of ASK cells exposed to APE and Doxorubicin for 48 h. Cells were lysed and DNA was extracted and electrophoresed on 1.5% agarose gels and stained with SYBER GOLD for detection of DNA fragmentation.

On the other side, high-grade gliomas have highly-vasculature that allows blood to nourish tumors. The disruption of blood supply is a strategy to kill the tumors. Andrographolide and ethanolic extract treated tumors would selectively inhibited tumor capillary sprouting without damaging the pre existing vasculature (Sheeja *et al.*, 2007). Thus, APE which contains andrographolide may inhibit tumor growth by decrease blood supply. In addition, the immune-stimulating of Andrographis paniculata make it an ideal candidate in cancer therapies without any major side effects. Mice studies have shown that Andrographolide and ethanolic extract stimulated both antigen specific and non-specific immune system. Similarly, the methanolic extract and purified diterpene andrographolides showed both anticancer and the immunostimulatory activity by enhance the IL-2 induction in human peripheral blood lymphocytes (Kumar *et al.*, 2004).

Herbal medicine of APE has been considered as a safe medicine in Chinese, India and Thailand. The acute and chronic toxicity of Andrographis paniculata in mice study that received oral extracts of at 15 g/kg body weight showed no evidence of organ damage and none of the mice died (Sithisomwongse *et al.*, 1989). Therefore, the dose used in the present study seems safe in terms of toxicity. Although Andrographis paniculata are much less toxic than most chemotherapeutic agents used to fight cancer, it may be seeing natural remedies combined with synthetic chemotherapeutic compounds that might improve efficacy and decrease side effects. The Andrographis paniculata is an inexpensive and easily obtained, so it would benefit many, especially people in developing countries where cancer is almost catastrophic. Additionally, the standardization of the active compounds in AP extracts need to perform further investigation.

Conclusion

In conclusion, APE showed an antiproliferative effect of ASK cancer cells via apoptotic pathway. APE was able to induce membrane blebbing, chromatin condensation and fragment of nuclear DNA. Also, APE is known for low toxicity in mice. As apoptosis has become a new therapeutic target in cancer research, these results confirm the potential of APE as anti-cancer compounds in glioma.

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