Full Length Research Paper

Eupatilin: A flavonoid compound isolated from the artemisia plant, induces apoptosis and G2/M phase cell cycle arrest in human melanoma A375 cells

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Eupatilin is a flavonoid compound isolated from the Artemisia plant. Eupitillin possesses antioxidant as well as potent anticancer properties. In this study, for the first time, we examined the anti-proliferative effects of Eupatilin in human melanoma A375 cells and its ability to induce apoptosis and cell cycle arrest. Eupatilin specifically induced morphological changes in A375 cells and was less toxic to the normal mouse splenocytes. The inhibitory effects of A375 cells were associated with the DNA damage, apoptosis, and cell cycle arrest at G2/M phase in a dose-dependent manner. The apoptotic effects of Eupatilin were further verified by using Annexin V-FITC/propidium iodide staining in flow cytometry. These results suggest that Eupatilin is an effective natural compound that worth further mechanistic and therapeutic studies against human melanoma.

Key words: Eupatilin, melanoma, apoptosis, cell cycle arrest.

INTRODUCTION

Melanoma, a malignant and aggressive tumor has continuously increasing incidence all over the world. Its chemotherapeutic treatment is widely studied by using single and/or combination of anticancer drugs to prevent the metastasis spread (Gray-Schopfer et al., 2007). The chemotherapeutic treatment of melanoma is done by using several compounds as single or in combinations (dacarbazine, cisplatinum and vindesine, response rate of 40%, including complete response of 4%) (Ives et al., 2007; Legha et al., 1996), but these chemotherapeutic treatments were not potent enough against melanoma. Developments of novel compounds that can prevent melanoma are in progress (Hofmann et al., 2007). In the past decades several natural compounds have shown a

great promise in treatment of cancer and numerous natural compounds of dietary and botanical origin has been identified with chemopreventive potential (Amin et al., 2009; Kintzios and Barberaki, 2004).

Eupatilin (5,7-dihydroxy-3',4', 6-trimethoxyflavone) is a flavonoid compound isolated from Artemisia plant. Recently Choi et al. (2009) has reported the novel antitumor, cytotoxic activity of Eupatilin through robust induction of apoptosis in human gastric carcinoma AGS cells complementing the previous findings by Kim at al. (2005b) and Lee et al. (2008). Similar inhibitory effects were also documented by Kim al. (2005a) during their studies on ras transformed breast epithelial cells (MCF-10A-ras). Antitumor potential of Eupatilin is also reported by Seo and Surh (2001) in leukemia HL60 cells, (Koshihara et al., 1983) in mast tumor and (Nam et al., 2008) in colon cancer cells. However, none of the previously published reports have shown an activity of Eupatilin against melanoma. Thus, in this study, for the first time we have evaluated the activity of Eupatilin on human melanoma cell line A375. This study will pave the

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way for further mechanistic exploration of this natural compound against melanoma.

MATERIALS AND METHODS

Chemical and reagents

Fetal bovine serum Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. DMEM culture medium, Methylthiazolyldiphenyl-tetrazolium MTT, Propidium iodide (PI), Hoechst, Trypan blue dye, and Dimethyl sulfoxide (DMSO) were purchased from Sigma.Annexin-V FITC apoptosis detection kit was purchased from Beyotime Institute of Biotechnology Jiangsu China.

Extraction, isolation, and identification of eupatilin

Eupatilin was isolated from Chinese herbal plant Artemisia through fractionation of Artemisia extract. Briefly, the herbal plant was crushed and extracted in Soxhlet extractor with alcohol for more than 12 cycles to achieve maximum extraction of its ingredients. The ethanol extract was hemi dried using rotary evaporator and then dissolved in 80% methanol. After centrifugation at 12000 rpm for 15 min, the supernatant was separated and filtered with 0.18 µm filter paper. Starting from the first peak to the end of the last peak, the extracted material was divided into 80 fractions on the basis of time (30 s per fraction) using high performance liquid chromate-graphy (HPLC). The fractions were dried and dissolved in dimethyl sulfoxide (DMSO) to obtain a 1 mg/ml stock solution. These fractions were subjected to screening for cytotoxicity against human melanoma A375 cells, analyzed by MTT assay and cells were microscopically examined to detect morphological changes. Materials around the positive fractions were collected and further fractionated by preparative HPLC (Solvent phase: Methanol and water with 0.02% acetic acid). Single positive compound was purified by further fractionation guided by anti-melanoma activity. An active single compound with 99.6% purity was characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR) to determine its chemical structure.

Cell culture

Human melanoma cell line (A375) was purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% FBS and 100 unit of Penicillin at 37°C in a CO_2 incubator with 5% CO_2, 95% air and 100% humidity. Cells were plated in 10 cm culture dish and allowed to grow to approximately 70% confluence before experimentation.

Cell proliferation assay

The growth inhibitory effect of the Eupatilin on the viability of cells was determined by the MTT assay. Briefly, A375 cells were plated at a density of 1×10^4 cells per well in 96-well plates. After 20 h, cells were treated with 100 µl of complete culture medium containing 25, 50, 100, 200, 400 and 800 µM eupatillin with equal amount of DMSO as negative control. Each concentration of Eupatilin was repeated in four wells. After incubation for 24 h, cell viability was determined. 10 µl MTT (5 mg/ml) in phosphate buffered saline was added to each well and incubated for 4 h. After removal of the medium, 150 µl DMSO was added to each well and shaken carefully. The absorbance was recorded on the microplate reader (ELX 800, BIO-TEK Instruments Inc.) at a wavelength of 570 nm. The inhibitory effect of Eupatilin on cell growth was assessed and

inhibition ratio (I%) was calculated using the following equation (Yu et al., 2007):

$$I\% = [A_{570} \text{ (control)} - A_{570} \text{ (treated)}] / A_{570} \text{ (control)} *100$$

Analysis of toxicity on murine splenocytes

In order to observe cytotoxic effect of Eupatilin on normal cells, splenocytes was isolated from CD1 mouse. Briefly, mouse was euthanized by overdose of pentobarbital. Spleen was surgically removed and mashed using the plunger end of the syringe in cold PBS buffer. Cell suspension was centrifuged at 1500 g for 5 min and pellet was resuspended in 1 ml of DMEM medium. Red blood cells were lysed with lysis buffer (0.01 M $KHCO_3$ to and 0.15 M NH_4Cl) for 40 s then 9 ml of medium was added and re-centrifuged. Supernatant was discarded again and pellet was resuspended in DMEM medium with 10% FBS. Cells were plated in 96 well plates at 20, 00 cells/well and treated with 150 and 300 µM Eupatilin. After 24 h incubation, cells were stained with 0.4% trypan blue, observed and photographed under microscopy.

Determination of apoptosis

The apoptotic rate of A375 cells was detected using flowcytometry with the AnnexinV-FITC/PI double labeling method. Briefly, A375 cells in logarithmic growth phase were plated in 6-well plates and allowed to attach overnight. Cells were treated with 150 and 300 µM of Eupatilin for 24 h. Then cells were collected, washed and resuspended in PBS. Apoptotic cell death was identified by double survival staining with recombinant FITC (fluorescent isothiocyanate)-conjugated Annexin V and PI, using the Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology, Hainan, Jiangsu, China) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed in flow cytometry using Cell Quest software. DNA fragmentation with the Hoechst staining was done following Ioannou and Chen (1996).

Cell cycle analysis

A375 cells were seeded in 12-well plates and then treated with 150 and 300 µM Eupatilin for 24 h. After treatments, the percentages of cells in the different phases of cell cycle were evaluated by determining the DNA content after propidium iodide staining. Briefly, cells were washed with PBS, trypsinized and centrifuged at 1000 rpm at 4°C for 5 min. Pellets were fixed overnight in 70% chilled ethanol. After fixation, cells were washed twice with PBS and incubated in PBS containing 10 µL/100 ml RNase (1 mg/ml) for 10 min at room temperature. Finally, samples were stained with 5 µL/100 ml of propidium iodide (1 mg/ml) for 30 min at 4°C. Data acquisition was done by flow cytometry (EPICSXL-MCL, Beckman Coulter, US) using Cell Quest software.

RESULTS AND DISCUSSION

The present study started with the screening of ethanol extract of Artemisia plant and led to isolation of target compound by using HPLC. The analytical HPLC chromatograph of ethanol extract is shown in Figure 1A. The single compound with inhibitory activity on A375 cells was isolated by subfractionation on preparative HPLC. The purity of single compound was determined by chromatograph that is shown in the Figure 1B. Compound
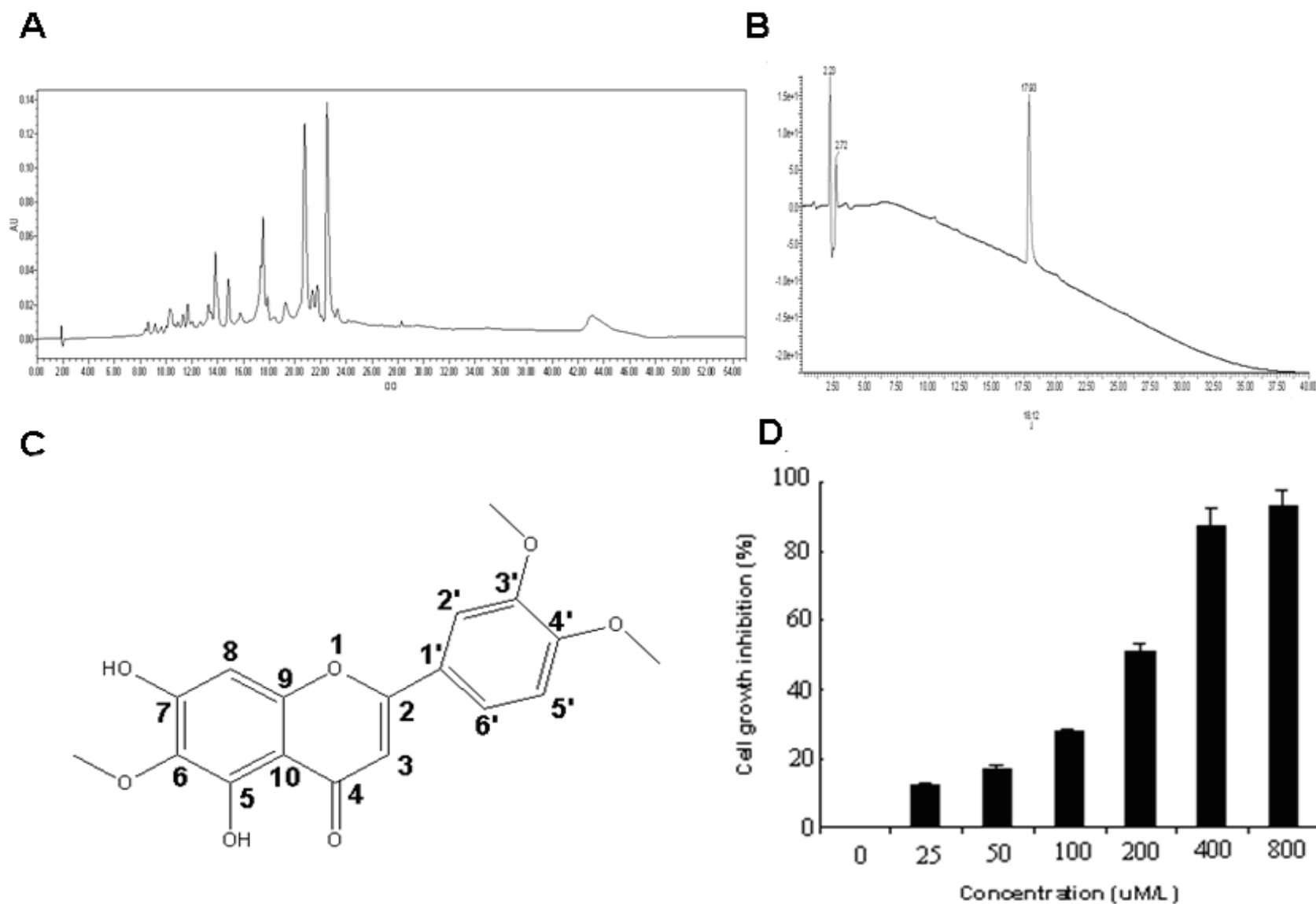
Figure 1. Isolation of natural compound Eupatilin and its cytotoxic effects: Analytical HPLC chromatograph of raw ethanol extract of Artemisia plant and single compound (A and B), structure of compound (C), cell viability was determined by MTT. The effect of Eupatilin on the cell growth inhibition of A375 cells compared with control. Actively dividing cells were treated for 24 h with different concentrations of Eupatilin varying from 0 to 800 μmol/L. Values are means, with standard deviations represented by vertical bars (D).
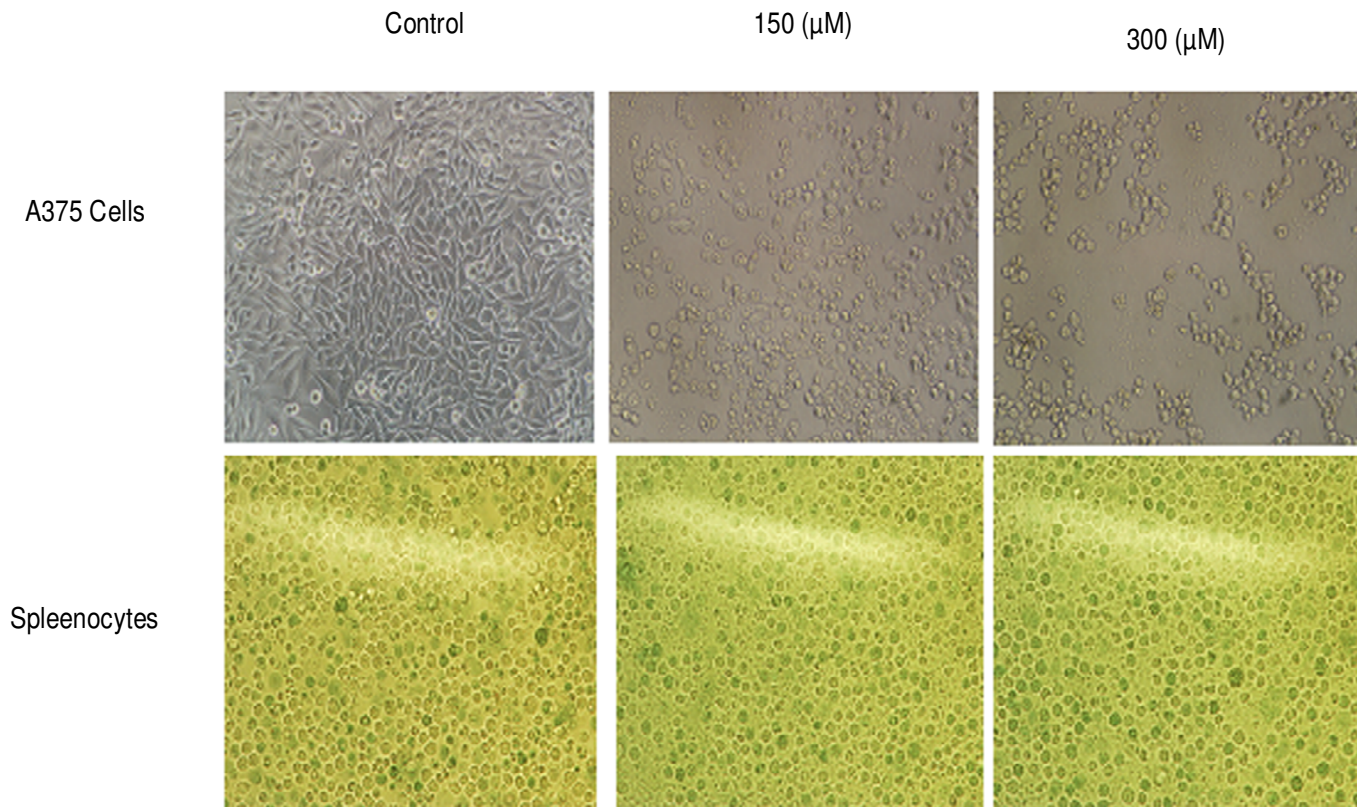
Figure 2. Morphological changes of melanoma A375 cells and splenocytes after treatment with 0, 150 and 300 µM of Eupatilin.

was identified by comparing its physical, spectroscopic (^{1}H NMR, ^{13}C NMR) and MS data with those that were reported in the literature (Kang et al., 2008). The structure of the com-pound is determined as Eupatilin as shown in the Figure 1C.

Cell growth inhibition of A375 melanoma cells by eupatilin

A375 human melanoma cells were treated with different concentrations of Eupatilin (0, 25, 50, 100, 200, 400, and 800 µM) for 24 h. Cell growth inhibition was measured by MTT assay. A dose-dependent increase of cell growth inhibition was observed as shown in Figure 1D. The effects of the Eupatilin on other malignant cell lines were reported previously. One study was done by Lee et al. (2008) and the other by Kim (2005) and associates reported the cytotoxicity of Eupatilin on gastric cancer (Kim et al., 2005b; Lee et al., 2008). Other studies also reported cytotoxic activity of Eupatilin against breast cancer (Kim et al., 2005a), leukemia (Seo and Surh, 2001), mast tumor (Koshihara et al., 1983) and colon cancer cells (Nam et al., 2008; Schulze-Osthoff et al., 1994; Wyllie, 1980; Zhang and Xu, 2000). Our study is the first report of the cytotoxic activity by Eupatilin against melanoma.

Effect of eupatilin on morphology of A375 melanoma cells and spleenocytes

Eupatilin induced typical morphological changes of cell death in A375 cells. It had little effect on spleenocytes at the same concentrations (Figure 2). The results clearly indicated that Eupatilin was less toxic for normal cells than melanoma cells.

Induction of apoptosis in A375 melanoma cells by eupatilin

Apoptosis is vital process for maintenance of homeostasis and eradication of damaged cells. There are many chemopreventive agents, which result in cancer cell cell death by induction of apoptosis (Kelloff et al., 2000). Previous studies have shown that Eupatilin induces potent growth inhibition in many tumor cell lines (Kim et al., 2005b; Lee et al., 2008). It was reported that Eupatilin induced apoptosis of MCF10A-ras breast cancer cells. The mechanistic studies indicated that Eupatilin increases the ratio of proapoptotic Bax to the anti-apoptotic Bcl-2 in a dose-dependent manner (Kim et al., 2004) and regulates the Raf/MEK/Erk signaling transduction pathway (Kim et al., 2007).

In the present study, Eupatilin inhibited the cell growth
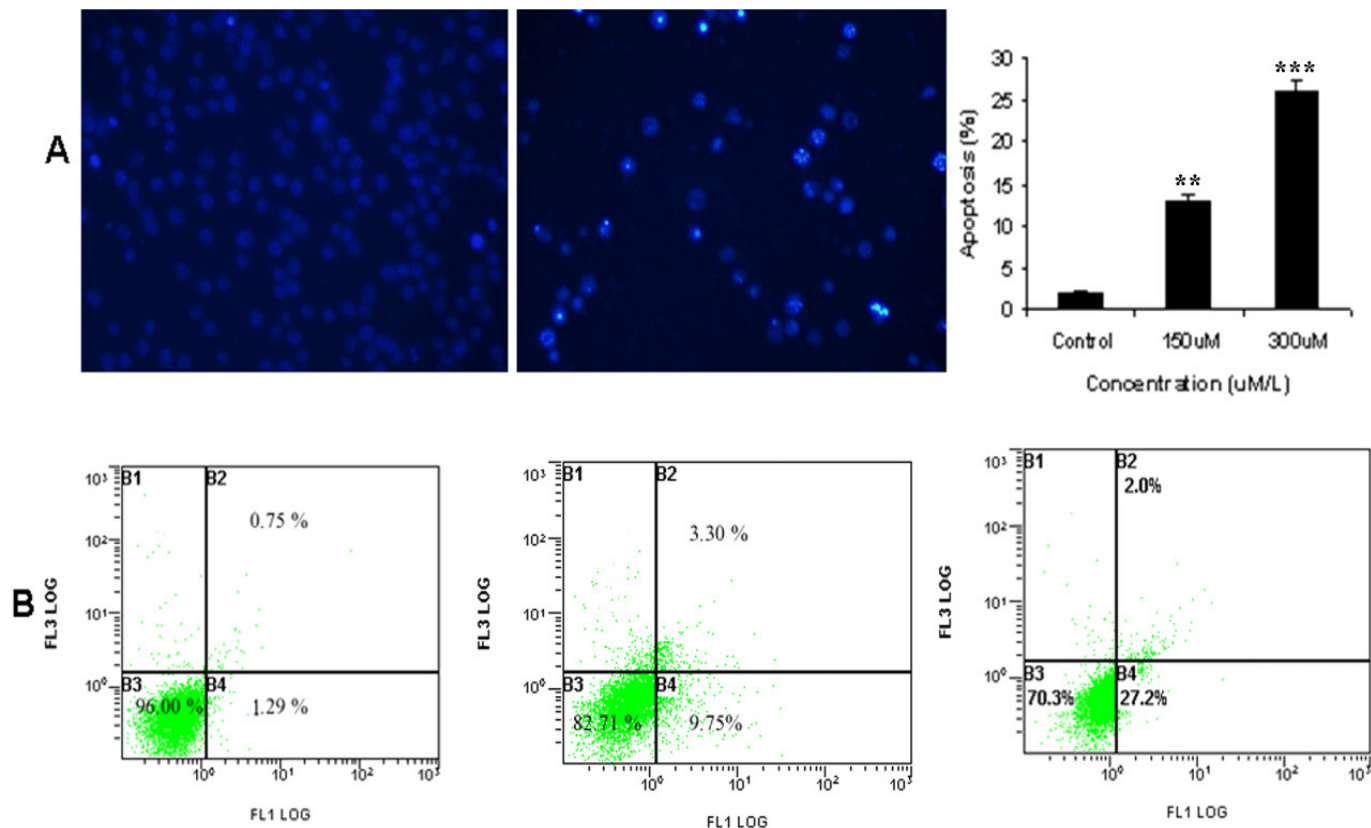
Figure 3. Apoptosis induced by Eupatilin in A375 melanoma cells: DNA damage and apoptosis in A375 cells was measured using the Hoechst staining under a fluorescence microscope after incubation with Eupatilin. Apoptotic cells showed as clumped or fragmented chromatin. Mean values of at least 100 cells calculated in each dose for Hoechst staining and Cell death after Eupatilin exposure (3A). Apoptosis was evaluated after treating A375 cells with 150 and 300 uM of Eupatilin staining with FITC annexin-V/PI at 24 h. Flow cytometry profile represents Annexin-V-FITC staining in *x* axis and PI in *y* axis. The number represents the percentage of apoptotic cells in each condition (3B). Three independent experiments for FITC annexin-V/PI are shown ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, compared to the negative control group.

of A375 cells. These data suggested that Eupatilin may be further examined as an effective chemopreventive agent against human melanoma by inducing apoptosis. Characteristic morphological apoptotic changes, including chromatin condensation, cell shrinkage, nuclear fragmentation, formation of apoptotic bodies, and DNA fragmentation were also detected in A375 cells treated with Eupatilin. The cell death due to apoptosis is indicated by fragmentation of DNA in cell nucleus (Schulze-Osthoff et al., 1994; Wyllie, 1980; Zhang and Xu, 2000), which is easily detected with the Hoechst staining (Ioannou and Chen 1996). As shown in Figure 3, Eupatilin treatment induced typical morphological changes of DNA fragmentation. Drug-induced cell death could be associated with apoptosis and necrosis (Kanduc et al., 2002; Kerr et al., 1972). To determine whether Eupatilin induces apoptosis or necrosis, we analyzed the rate of apoptosis induced by Eupatilin in A375 cells by double staining with FITC annexin-V and propidium iodide (PI). It was observed that the apoptosis rates were 13.05 and 29.2% in the cells treated with 150 and 300 μM

eupatilin for 24 h respectively compared to the control cells at 2.04% (Figure 3B). Previous studies also reported the induction of apoptosis by Eupatilin in human gastric cancer (AGS) cells (Kim et al., 2005b; Lee et al., 2008) which is consistent with the result of the present study.

Eupatilin induces cell cycle arrest of A375 melanoma cells

It is reported that flavonoid has the potential to arrest cell cycle in a variety of cancer cells (Auyeung and Ko, 2010; Drees et al., 1997; Priyadarsini et al., 2010; Wang et al., 2000). Several studies have indicated that for persistence of a normal cell cycle, cells undergo certain cell cycle check points for the surety of proper accomplishment of different events of cell cycle and for the protection of dividing cells as a result of severely damaged DNA (Singh and Khar, 2006; Tse et al., 2007). To further clarify the mechanism of growth inhibition by Eupatilin, flow cytometry was performed to determine whether eupatilin
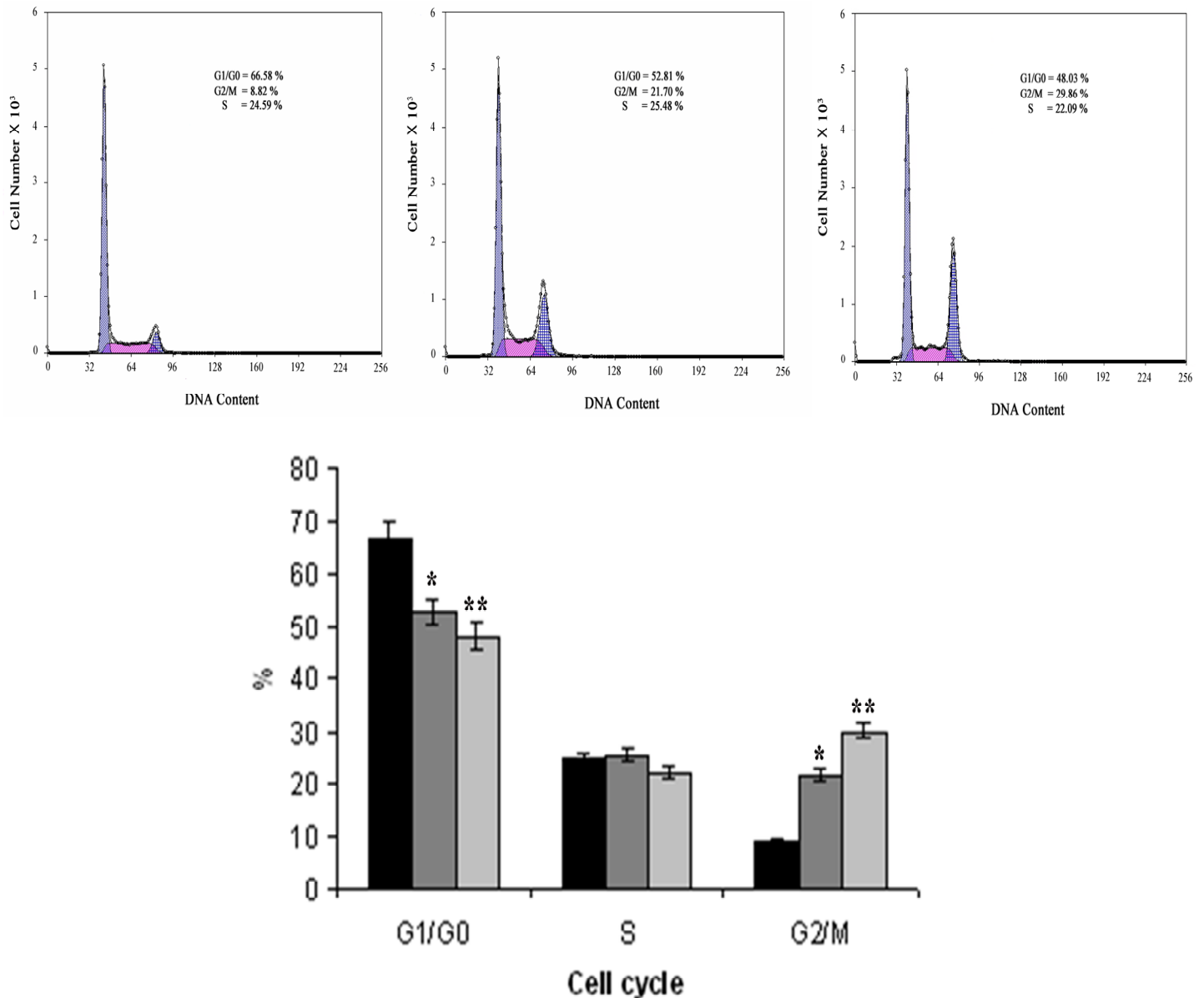
Figure 4. Cell cycle arrest of A375 cells by Eupatilin. Cell cycle distribution in A375 cells treated with 0, 150 and 300 µmol/L Eupatilin for 24 was analyzed by flow cytometry. ***P<0.001, **P<0.01, *P<0.05, compared to the negative control group (n=3).

induced cell cycle arrest. A375 cells were treated with 150 and 300 µmol/L of Eupatilin for 24 h. The results showed that Eupatilin arrested the cell cycle of A375 cells in the G_2/M phase. The percentage of G_2/M phase were increased from 8.82% in untreated cells to 21.70, and 29.86% in the cells treated with 150 and 300 µM eupatilin for 24 h, respectively. The ratio of G_2/M phase significantly increased in the Eupatilin treated groups compared to the negative control group and was accompanied by a decrease in cell population in the G_1/G_0 phase of the cell cycle in A375 cells (Figure 4).

These results revealed that cell cycle arrest at G2/M phase is one of the mechanisms by which Eupatilin

inhibits the proliferation of A375 cells. This study also demonstrated that Eupatilin arrests the cell cycle as a result of the DNA damage and indicated that Eupatilin induced growth inhibition is likely to involve the modulation of cell cycle progression in A375 cells. During DNA damage, cells are blocked in G2/M phase to provide time to repair damaged DNA (Molinari 2000; Abraham, 2001), or lead to apoptotic cell death in case of severe DNA damage (Huang et al., 2008).

Previous studies reported that Eupatilin induces the cell cycle arrest at different phases. For example, Eupatilin induced gastric carcinoma AGS cells at G1 phase (Choi et al., 2009). The result of Eupatilin in inducting cell cycle

arrest at G2/M phase is also consistent with other studies that some other flavonoid compounds arrest cell cycle at G2/M phase (Auyeung and Ko, 2010; Priyadarsini et al., 2008).

In conclusion, the results of present study indicated that Eupatilin inhibited cell proliferation of A375 melanoma cells by inducing apoptosis. The induction of apoptosis was partly regulated by arresting cell cycle at G2/M phase. These *in vitro* results suggest that Eupatilin should be further examined for its therapeutic value in human melanoma.

ACKNOWLEDGEMENTS

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