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Full Length Research Paper

Antiproliferation and apoptosis induced by Bisdemethoxycurcumin in human ovarian cancer cell SKOV3

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Bisdemethoxycurcumin, an active ingredient from the rhizome of the plant, *Curcuma longa*, has antioxidant, anti-inflammatory and anti-cancer activities. It has recently been demonstrated that the anticarcinogenic activities of Bisdemethoxycurcumin might be due to its ability to inhibit cell growth and induce apoptosis. In the present study, we have investigated the effects of Bisdemethoxycurcumin on growth and apoptosis in the human ovarian cancer cell line SKOV3 by MTT assay, fluorescence microscopy, flow cytometry and Western blotting. Our data revealed that Bisdemethoxycurcumin could significantly inhibit the growth and induce apoptosis in SKOV3 cells and a decrease in expression of Bcl-2, Bcl-XL and NF-κB. These activities may contribute to the anticarcinogenic action of Bisdemethoxycurcumin.

Key words: Bisdemethoxycurcumin, apoptosis, ovarian cancer, SKOV3 cells.

INTRODUCTION

In recent years, the morbidity and mortality of cancer still reaches a high plateau and is a major public health problem worldwide. Ovarian cancer represents the fourth leading cause of cancer-related death for women in the Western world (Jemal et al., 2008). The main reasons for the low survival rate of patients with ovarian cancer are the lack of effective early detection technology and treatment methods, as well as the highly metastatic nature of the disease. While most solid tumors metastasize through blood vessels or lymphatic nodes, ovarian cancer sheds cells into the peritoneal cavity (Hennessy and Markman, 2009). Searching for new compounds for the treatment of cancer is the aim of numerous studies, and many works are focused on plant-derived compounds that have curative potential and have been used widely in traditional medicines.

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Bisdemethoxycurcumin (Figure 1) is a phenolic compound with yellow color from the plant, Curcuma longa, which has been used as a kind of traditional Asian medicine for centuries. Bisdemethoxycurcumin has been reported for its anti-oxidant and anti-inflammatory activities (Jayaraj et al., 2010). However, the mechanisms underlying the anti-tumor abilities of Bisdemethoxycurcumin are still not clear enough. Therefore, it is critical to search for novel anticarcinogenic agents that can inhibit the growth or induce apoptosis of ovarian cancer cells. The present study was conducted to assess the anticarcinogenic effects of Bisdemethoxycurcumin on the human epithelium ovarian cancer cell line SKOV3, *in vitro*.

MATERIALS AND METHODS

Chemicals

Bisdemethoxycurcumin (Sigma) was dissolved in dimethyl sulfoxide(DMSO) at a concentration of 10 mM and stored in a dark-colored bottle at –20ºC as a stock solution. The stock was diluted to

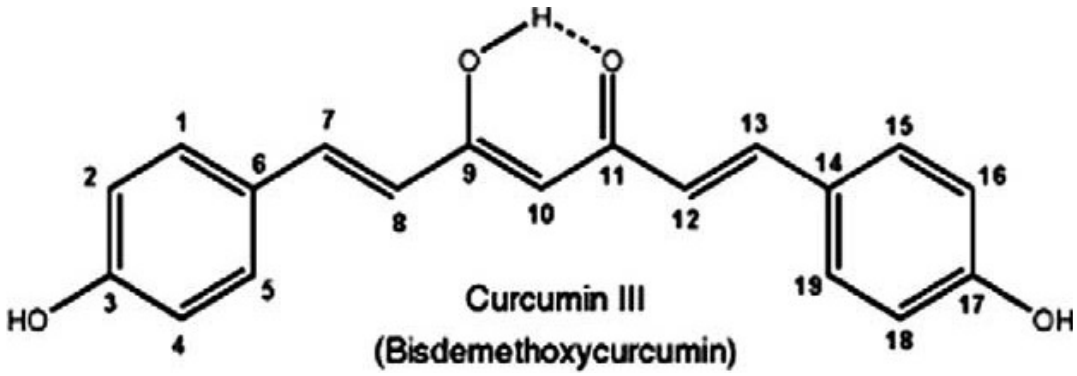

Figure 1. Structures of Bisdemethoxycurcumin used in the present study.

the required concentration with serum-free medium immediately before use. Antibodies against Bcl-2, Bcl-XL, NF-κB and were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines

SKOV3 human ovarian cancer cells were grown in 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated FBS. Cultures were maintained at 37℃ in a 5% CO_2/ 95% air atmosphere.

Morphology

Morphology of cells and the growth of good SKOV3 5 µM, 10 µM, 15 µM BDMC were incubated for 24 h; cells were collected, PBS washed, supernatant was observed under inverted microscope morphological changes, photo imaging.

MTT assay for cell viability

SKOV3 cells were seeded into 96-well plates at a density of 4×10^3cells/well in 200 µl of medium. After 24 h of culture, culture supernatant was removed and serum–free 1640 containing various concentrations of BDMC was added and incubated for 6,12,24 h. At each time point, 20 µl MTT (5 mg/ml) was added to each well and the cultures were incubated for an additional 4 h at 37℃. Culture medium was then removed and the formazan crystals dissolved by the addition of DMSO (150 µL/well). Absorbance was measured at 570 nm using an ELISA plate reader. All experiments were performed at a minimum of 3 times.

Cell growth inhibition rate = 1 - (experimental group OD value /control group OD value) × 100%

Immunohisto chemistry

Growth of SKOV3 cells was 1×10^5; the treated cells were seeded into a small glass slide containing 24 well plates, with 3 holes. Each hole (100 µl) was placed in an incubator of 37℃, having 5% CO_2 conditions, and cultured for 24 h. The cells adhered, the medium was changed to the hole when added at final concentrations of 5, 10, 15 µM bisdemethoxycurcumin. This continued for foster 24 h, and was washed with PBS for 5 min twice. Cold acetone was stored at 4℃, fixed for 15 min, and then the detection of PCNA by immunohisto chemistry. Immunohistochemistry SP methods include: mounting of fixed convention and washed with PBS. Then the volume fraction of 3% H_2O_2 at room temperature was incubated for 10 min in a distilled water; PBS was washed; normal goat serum fluid was enclosed at a room temperature and incubated for 20 min. There was dumping of serum, 1:100 anti-dropping PCNA, monoclonal antibody (primary antibody) in wet boxes of 4℃ overnight. PBS was washed. Biotin-labeled secondary antibody working solution was kept in wet boxes at 37℃ for 30 min; PBS wash; peroxidase labeled streptavidin working solution was dropped and incubated at room temperature for 30 min. DAB Reagent color was used; and also tap water, Hematoxylin, routine dehydration, clearing. PBS was used instead of primary antibody as control. The remaining steps are the same. There were positive results for the brown particles inside a nucleus; the negative control had no such particles appear.

Hoechst 33258 staining

SKOV3 cells in the process of apoptosis would show significant morphological changes in the nuclear chromatin, which can be revealed by Hoechst 33258 staining. In this assay, cells were seeded on cover slips in the 6-well plate and treated with 5, 10, 15 µM bisdemethoxycurcumin. After 24 h, the cover glasses were washed carefully with PBS and stained with 20 mg/ml Hoechst 33258 for 10 min. Thereafter, the cells were washed in PBS, and observed by an Olympus IX51 microscope equipped with a DC300F camera.

Flow cytometric cell cycle analysis

To determine the effect of Bisdemethoxycurcumin on the cell cycle, SKOV3 cells were treated with 5, 10, 15 µM for Bisdemethoxycurcumin at 24 h, respectively. They were washed, and fixed with 70% ethanol. After incubation overnight at -20°C, cells were washed with PBS and then suspended in staining buffer (propidium iodide, 10 mg/ml; Tween-20, 0.5%; RNase, 0.1% in PBS). Cell cycle was analyzed by flow cytometry (FACSAria, Becton Dickinson, USA) and the percentage of cells in various phases of cell cycle was calculated. Gating was set to exclude cell debris, cell doublets and cell clumps.

Indirect immunofluorescence study of NF-κB

Flow cytometry (FCM) was performed in two groups to detect the expression of NF-κB in SKOV-3 cells. Antibody against NF-κB was

purchased from Santa Cruz Biotechnology (USA). SKOV-3 cells were grown in parallel cultures with and without 5, 10, 15 μM Bisdemethoxycurcumin for up to 24 h under the same condition and then used for NF-κB assay. APC-labeled antibody was used as secondary antibody.

Annexin Ⅴ FITC / PI apoptosis assay

SKOV3 cells were seeded onto 6-well plates at a density of 1×10^5 cells/well in logarithmic growth phase. After 24 h of culture, culture supernatant was removed and serum–free (1640) containing various concentrations of BDMC was added and incubated for 24 h. Then cells were digested, collected and placed in a centrifugal suspension with a centrifuge which is 2000 rpm every 5 min; the supernatant fluid was discarded. Cell pellets were fixed with 70% alcohol, and washed for 3 times with PBS. Then the cell pellets were tested on a machine.100 μl binding buffer suspension was added into the cell suspension, and then added 10 μl Annexin Ⅴ-FITC and 5 ~ 10 μl of propidium iodide (PI) and mixing them together. They were incubated at room temperature and at the same time kept away from light for 15 min. After that, 400 μl binding buffer was into the mixture and tested by Beckman Coulter type's flow cytometry. The results were analyzed by EXPO32 ADC software. Finally, apoptosis value was recorded.

Western blotting

Cells were washed in PBS and lysed in boiling sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (62.5 mM Tris (pH 6.8), 1% SDS, 10% glycerol, and 5% β-mercaptoethanol). The lysates were boiled for 5 min, separated by SDS–PAGE, and transferred to an Immobilon membrane (Millipore). After nonspecific binding sites were blocked for 1 h using 5% skim milk, the membranes were incubated for 2 h with specific antibodies. Membranes were then washed three times with Tris-Buffered Saline Tween-20 (TBST) and incubated further for 1 h with horseradish peroxidase-conjugated anti-rabbit, -mouse, or -goat antibody. Visualization of protein bands was accomplished using ECL. (millpore,corporation,Billerica,USA). The respective protein band intensity was quantified by densitometric analysis using the Gel-pro Analyzer. Representative results from at least three independent experiments are shown.

Statistical analysis

Statistical analyses were performed using one-way ANOVA; $P<0.05$ or $P<0.01$ was considered statistically significant. All comparisons were made relative to untreated controls and significance of differences is indicated as $^{*}P<0.05$ and $^{**}P<0.01$. All statistical analyses were performed using SPSS 12.0 software.

RESULTS

Morphology

Morphology was observed under inverted microscope; cell growth controls skov3 active and cluster growth; cell size is consistent, being round or oval. The experimental group was treated with BDMC; in particular the role of 10 and 15 μM after 24 h decreased cells significantly at cytoplasmic concentration. Some cells were obviously smaller, irregular contour, with slow growth. With the increase of drug concentration and the role of time, cells significantly reduced cell death commonly (Figure 2).

Bisdemethoxycurcumin inhibits the proliferation of human ovarian cancer cell line SKOV3

SKOV3 cells were exposed to various concentrations of Bisdemethoxycurcumin for 6, 12 or 24 h, and their cellular proliferation was monitored. The result demonstrated that Bisdemethoxycurcumin inhibited the proliferation of SKOV3 cells. The anti-proliferative effects of Bisdemethoxycurcumin were both dose- and time-dependent (Figure 2A). A marked inhibition in cellular proliferation was observed in cells treated for 24 h with 15 μM. Bisdemethoxycurcumin was compared with untreated control cells.

The BDMC effect on the SKOV3 cells with different concentrations of PCNA

PCNA positive cells in the test results show that there was PCNA immunocytochemistry positive particles in the nucleus; in each drug group PCNA expression was significantly lower than the control group, and with the increase of drug concentration, PCNA expression decreased (Figure 2B)

Hoechst 33258 staining

Cells in the process of apoptosis would show significant morphological changes in the nuclear chromatin, which can be revealed by Hoechst 33258 staining. In this assay, cells were seeded on cover slips in the 6-well plate and treated with 5, 10, 15 μM Bisdemethoxycurcumin. After 24 h, the cover glasses were washed carefully with PBS and stained with 20 mg/ml Hoechst 33258 for 10 min. Thereafter, the cells were washed in PBS; fluorescence was analyzed using an Olympus IX51 microscope equipped with a DC300F camera (As shown in Figure 3).

Results of cell cycle analysis

Cell cycle of SKOV-3 cells cultured in two groups was analyzed using FCM (Figure 4). In each group, the percentage of SKOV-3 cells in G1, S and G2 phases was calculated, which was based on DNA content distribution histogram. The results displayed that the percentage of SKOV-3 cells cultured in the absence of Bisdemethoxycurcumin (group A) in G1 phase was lower than that cultured in the presence of Bisdemethoxycurcumin (group B-D) ($p<0.01$). However, the percentage of SKOV-3 cells cultured without Bisdemethoxycurcumin (group A) in S phase was higher

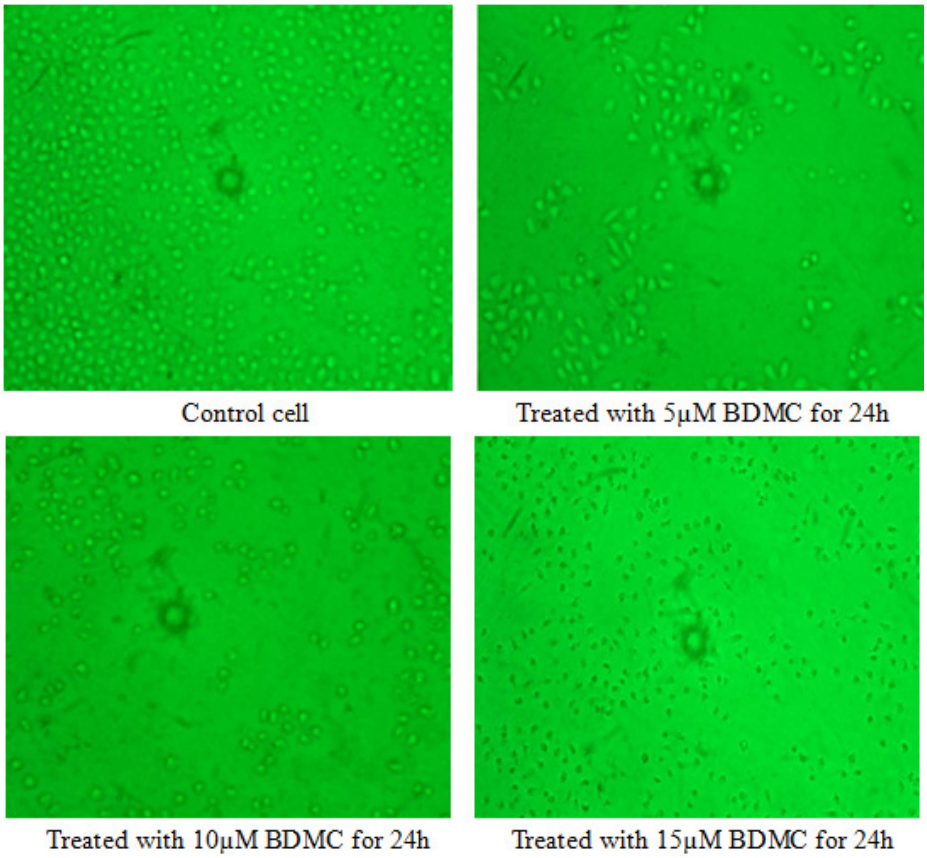

Figure 2. Effects of (Bisdemethoxycurcumin, BDMC) on growth profile of skov3 cells (×100). Note: i) skov3 cells in control group ii) (Bisdemethoxycurcumin, BDMC) of 5 μM for 24 h iii) (Bisdemethoxycurcumin, BDMC) of 10μM for 24h 4. (Bisdemethoxycurcumin, BDMC) of 15 μM for 24 h.

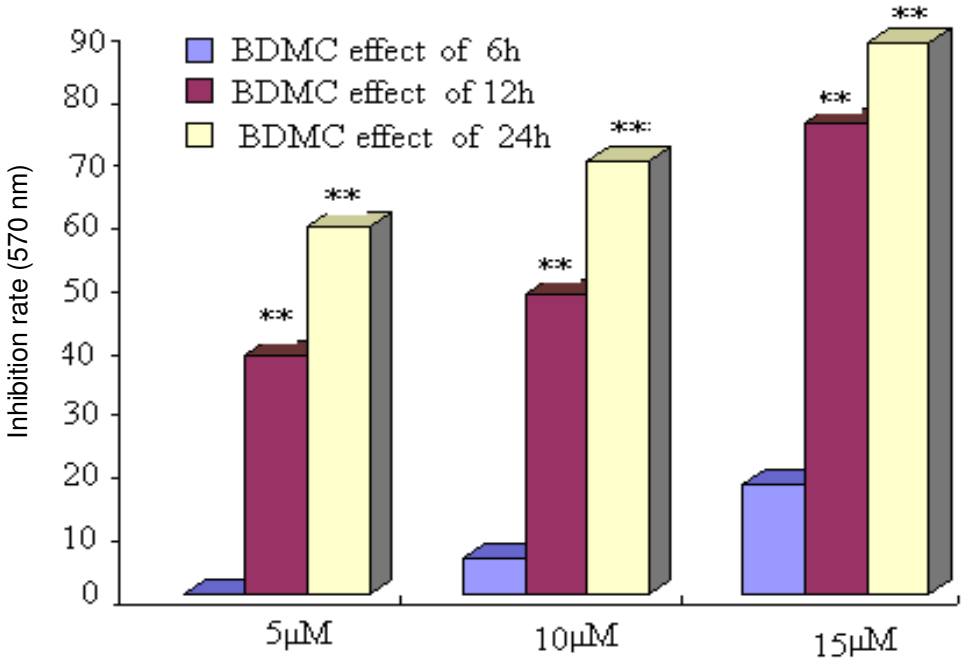

Figure 2A. The BDMC effect on the SKOV3 cells with different concentrations of PCNA.

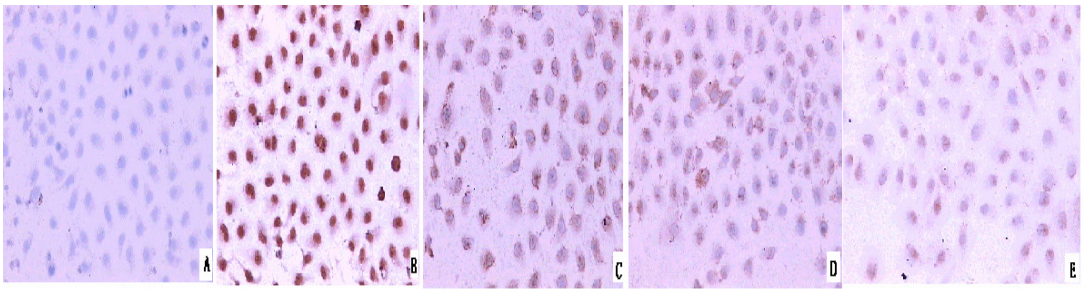

Figure 2B. The BDMC effect on the SKOV3 cells with different concentration of PCNA. Note: A: negative control B: skov3 cells in control group C: (Bisdemethoxycurcumin, BDMC) of 5 µM for 24 h D:(Bisdemethoxycurcumin, BDMC) of 10 µM for 24 h E: (Bisdemethoxycurcumin,BDMC) of 15 µM for 24 h.

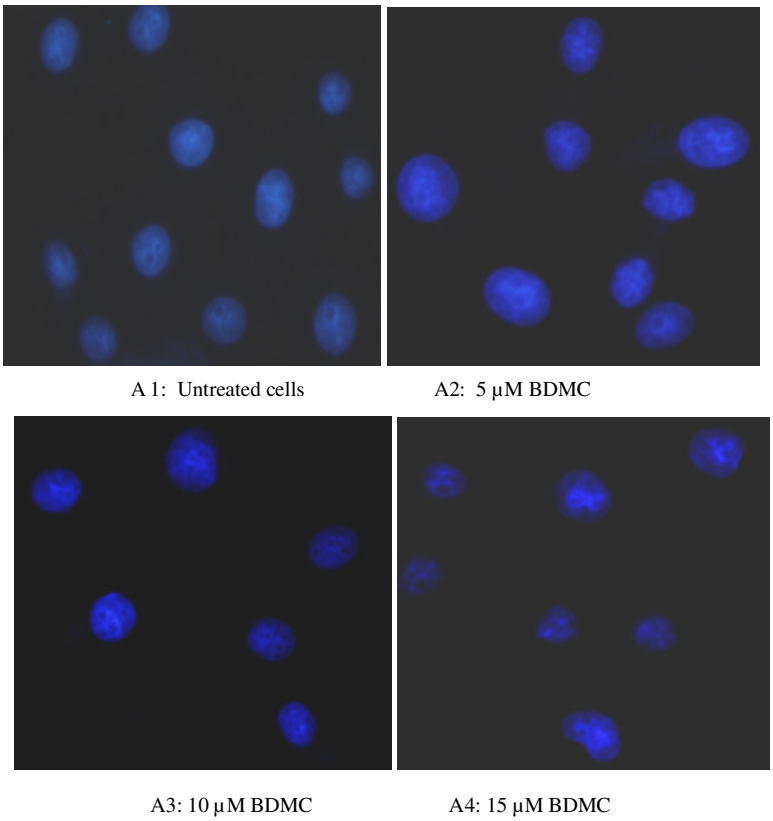

Figure 3. Hoechst 33258 fluorescent staining detection of apoptotic morphology in SKOV3 cells treated with 5, 10 and 15 µM Bisdemethoxycurcumin for 24 h. (A1) Untreated cells; (A2-4) cells treated with 5, 10 and 15 µM Bisdemethoxycurcumin for 24 h.

than that cultured with Bisdemethoxycurcumin (group B-D) ($p<0.01$).

NF-κB expression, Apoptosis analysis and Western blotting analysis

Results for NF-κB expression, Apoptosis analysis and Western blotting analysis are as shown in Figures 5, 6 and 7, respectively.

DISCUSSION

Natural curcuminoids have been used as a chemopreventive agent for a wide variety of cancers, including colon, pancreatic and breast cancers. (Kunnumakkara et al., 2008). Several studies have shown that three forms of curcuminoids have similar biological activities but different potency. Interestingly, in some cases, BDMC showed the highest potency of the three forms of curcuminoids, having antiproliferative

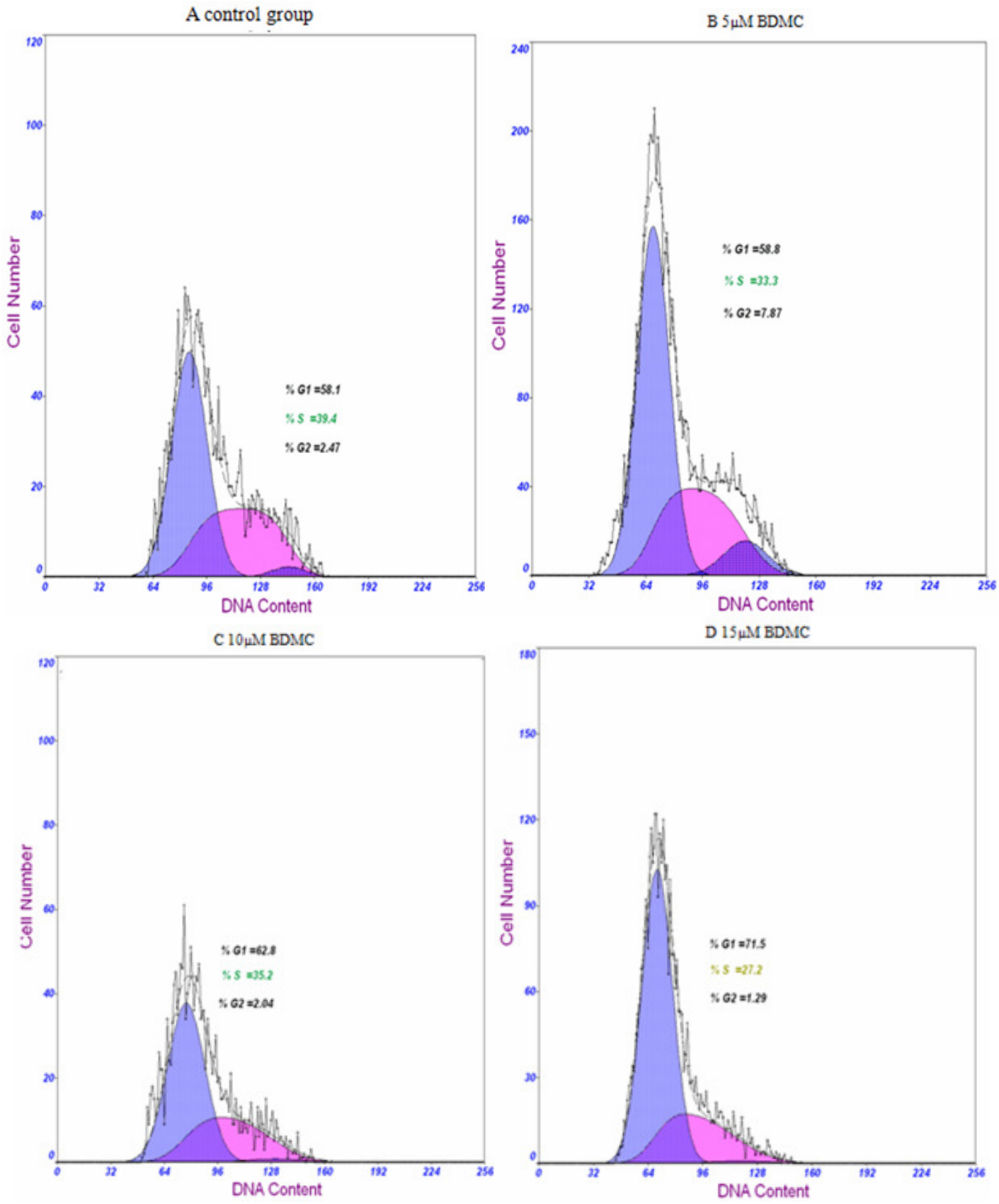

Figure 4. FCM results in groups of (A) and (B-D). Note: A: percentage of SKOV-3 cells cultured in the absence of Bisdemethoxycurcumin (group A) B-D: in the presence of Bisdemethoxycurcumin (group B-D).

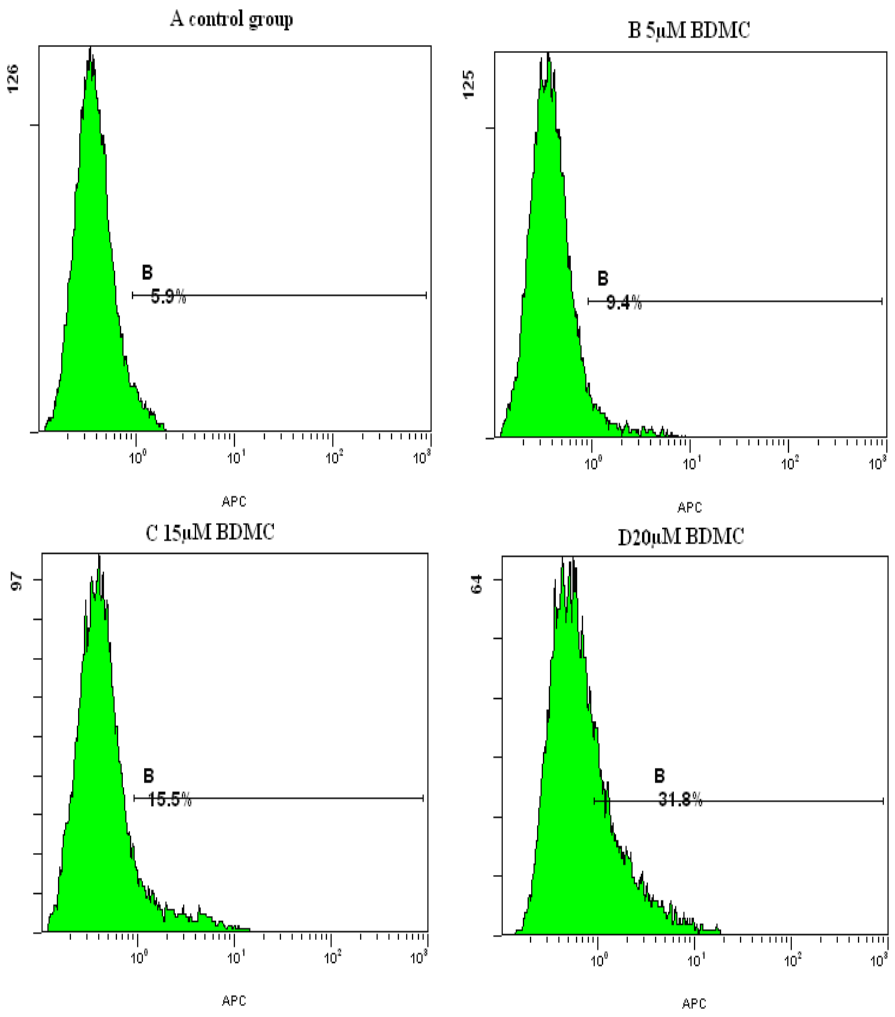

Figure 5. The BDMC effect on the SKOV3 cells with different concentration of NF-κB. Note: A. SKOV3 cells in control group B. (Bisdemethoxycurcumin, BDMC) of 5 μM for 24 h; C. (Bisdemethoxycurcumin, BDMC) of 10 μM for 24 h; D. (Bisdemethoxycurcumin, BDMC) of 15 μM for 24 h.

activity against MDA-MB-231 breast carcinoma cells (Yodkeeree et al., 2010), and to cause down-regulation of COX-2 and iNOS expression (Zhang et al., 2008). Moreover, it was found that BDMC inhibits HT1080 cell invasion with higher potency than curcumin, but not significantly different from that of bisdemethoxycurcumin (Yodkeeree et al., 2009).

In this study, the role of different concentrations of BDMC at different times using SKOV3 ovarian cancer cells to study the BDMC on the biological behavior of SKOV3 cells showed different concentrations of BDMC on ovarian cancer cell line SKOV3 significantly affect the shape, make cell condensation, smaller size, slow growth, decrease cell number, or even death. And with the increase of drug concentration and time of the extension, this effect is more apparent. MTT method confirmed the same could inhibit the growth of SKOV3 cells and showed a stress relief-a dose-dependent relationship.

PCNA which is required for cellular DNA synthesis of a protein is cell G / S on the synthesis of protein whose expression level may reflect the degree of tumor cell proliferation (Stalinska et al., 2009). The higher the degree of tumor cell proliferation, tumor growth is faster, more prone to transfer, the higher the degree of malignancy. Therefore, many scholars have already assessed PCNA expression as a useful indicator of tumor prognosis. (Chen et al., 2010). These results show 15 μM BDMC role in SKOV3 tumor cells after 24 h, as compared with the control group; BDMC treated cells were significantly decreased with PCNA expression, and increased with concentration of PCNA expression which gradually reduced. This suggests that BDMC can inhibit the expression of PCNA protein, play a role in anti-tumor, may be have effect against ovarian cancer, which is one of the targets.

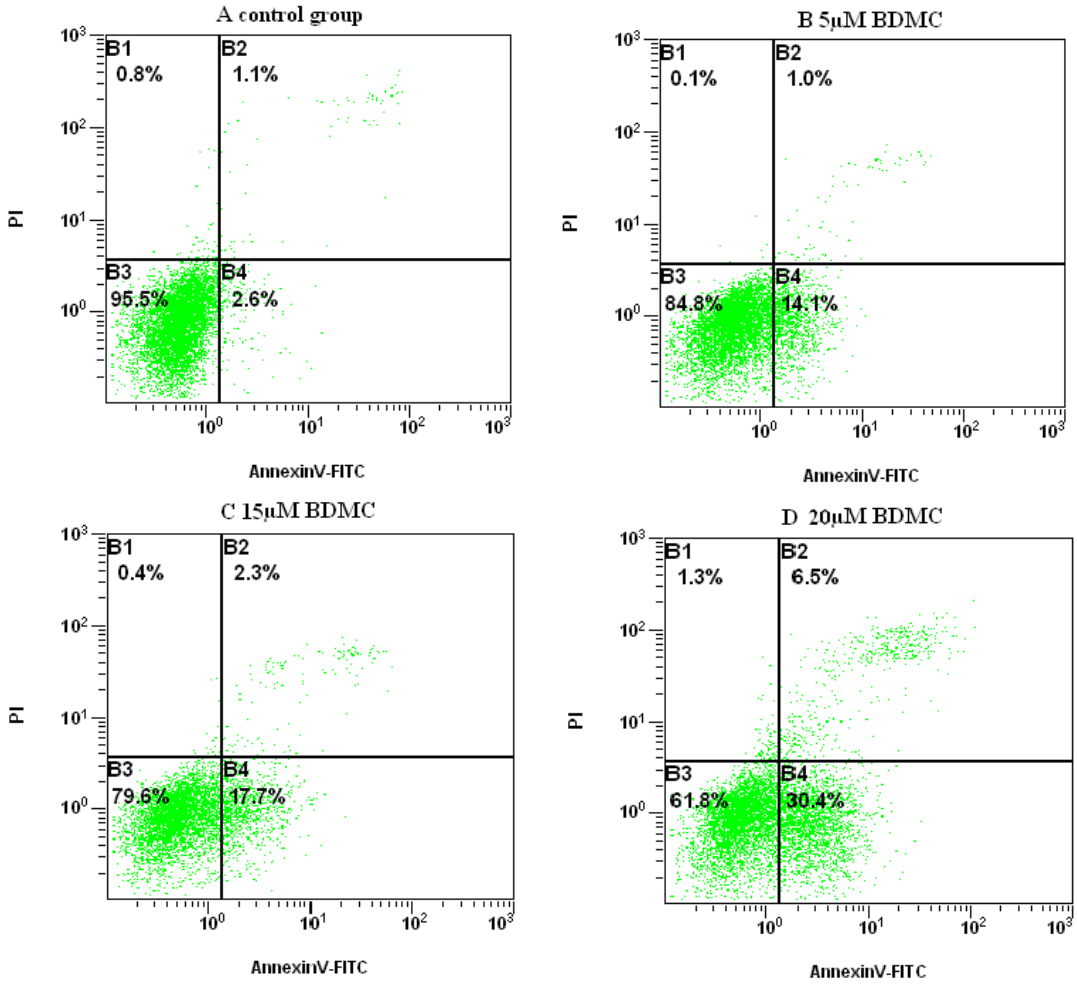

Figure 6. A. skov3 cells in control group B. (Bisdemethoxycurcumin, BDMC) of 5 μM for 24 h; C. (Bisdemethoxycurcumin, BDMC) of 10 μM for 24 h D. (Bisdemethoxycurcumin, BDMC) of 15 μM for 24 h. B1: the percentage of dead cells; B2: late apoptosis (death) cells; B3: percentage of living cells; B4: Percentage of early apoptotic cells.

NF-κB transcription factor is constitutively activated in many cancers, including breast cancer, and has been shown to contribute to the development and progression of tumors.

Many studies have shown that inhibition of NF-κB activity could suppress angiogenesis, invasion and metastasis by down regulating the expression of NF-κB downstream target genes, such as BCL-xl, Bcl-2 commonly known NF-κB consisting of a p50/p65 heterodimer (Jutooru and Chadalapaka, 2010). NF-κB is retained in the cytoplasm in an inactive form, bound by an inhibitory protein called inhibitor of κB (IκB) in most resting cells. The activation of NF-κB occurs as it is transported from the cytoplasm to the nucleus upon the degradation of the inhibitory subunit. In the nucleus, NF-κB binds to cognate sequence in the promoter region of many target genes. Therefore, NF-κB DNA binding activity is a hallmark for its activation (Shishir et al., 2005). Here, we found that treatment with BDMC of SKOV3 cells resulted in inhibition of NF-κB DNA binding activity in a dose–dependent manner (Figure 5). So Bisdemethoxycurcumin is a potent blocker of NF-κB activation, which has been linked with proliferation, invasion, and angiogenesis as well as induction of apoptosis.

In summary, our study demonstrates that Bisdemethoxycurcumin inhibits the proliferation of skov3 cells by arresting cell cycle and decreasing expression of Bcl-2, Bcl-XL and NF-κB and inducing apoptosis. Bisdemethoxycurcumin compared with cytotoxic drugs has wide variety of sources, low cost, no side effects, etc, and as an anti-mutagen and anti-tumor promotion agent, will have good prospects.

ACKNOWLEDGEMENT

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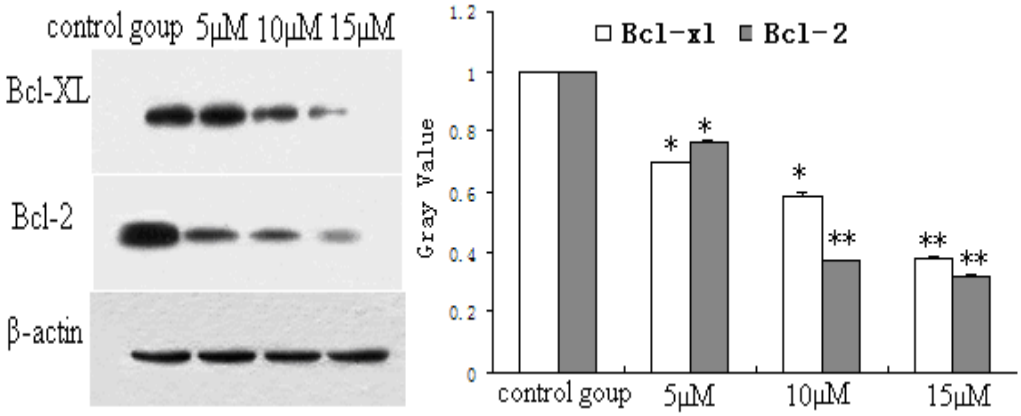

Figure 7. Effects of Bisdemethoxycurcumin on Bcl-XL and Bcl-2. skov3 cells were incubated (24 h, 37℃) in the absence and presence of various indicated concentrations (5–15 μM) of BDMC in serum-free medium. Equal amount of proteins was loaded (5μg/lane). The data represent the mean ± S.D of three independent experiments. Statistical analyses were performed using one-way ANOVA. **P<0.01 was considered statistically significant.

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