

3- β -Erythrodiol isolated from *Conyza canadensis* inhibits MKN-45 human gastric cancer cell proliferation by inducing apoptosis, cell cycle arrest, DNA fragmentation, ROS generation and reduces tumor weight and volume in mouse xenograft model

KAI LIU¹, YUE-HONG QIN², JIAN-YONG YU¹, HENG MA¹ and XI-LIN SONG¹

¹Department of Gastrointestinal Surgery, Shandong Tumor Hospital and Institute, Jinan, Shandong 250117;

²Department of Neurosurgery, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, P.R. China

Received October 30, 2015; Accepted December 11, 2015

DOI: 10.3892/or.2016.4610

Abstract. The objective of the present study was to investigate the *in vitro* and *in vivo* anticancer and apoptotic effects of 3- β -erythrodiol, a plant-derived triterpene against MKN-45 human gastric cancer cells. In addition, effects on cellular morphology, cell cycle phase distribution, DNA fragmentation, and ROS generation were also elucidated in the current research work. Cytotoxic activity of 3- β -erythrodiol was demonstrated by MTT cell viability and LDH assay. Cellular morphological study was carried out using phase contrast, fluorescence and scanning electron microscopy. Cell cycle analysis was evaluated by flow cytometry and gel electrophoresis was used to evaluate DNA fragmentation pattern. The results of the present study revealed that 3- β -erythrodiol induced dose-dependent as well as time-dependent anticancer effects in MKN-45 gastric cancer cells. Cellular morphological changes in MKN-45 cells as indicated by fluorescence and scanning electron microscopy were induced by 3- β -erythrodiol. This triterpene induced both early and late apoptotic features in these cancer cells. 3- β -Erythrodiol treatment led to sub-G1 cell cycle arrest with a corresponding decrease in S-phase cells and an increase in G2/M phase cells. DNA fragments were evident in gel electrophoresis experiment following 3- β -erythrodiol treatment. It was observed that 0.50 and 1.0 μ g/g 3- β -erythrodiol injection reduced the tumor weight from 1.4 g in PBS-treated group (control) to 0.61 and 0.22 g, respectively. Similarly, 0.50 and 1.0 μ g/g 3- β -erythrodiol injection reduced the tumor volume from 1.5 cm³ in PBS-treated group (control) to 0.91 and 0.31 cm³, respectively. The present investigation indicates

that 3- β -erythrodiol exerts anti-proliferative effects in human gastric cancer by inducing early and late apoptosis, cell cycle arrest, and ROS generation. It also decreased the tumor volume and tumor weight in male Balb/c nude mice.

Introduction

Gastric cancer is the most common malignant cancer of the gastrointestinal tract in the world. Gastric cancer is the second leading cause of mortality in the world after lung cancer. It is estimated that more than 750,000 new cases of gastric cancer are diagnosed every year throughout the globe (1). Gastric cancer has been found to be more prevalent in people aged 60 years or above. In 2005, the incidence rate of gastric cancer (0.3 million deaths and 0.4 million new cases) ranked third among the most common cancers in China (2). The actual cause of gastric cancer is unknown but it has been linked with low vitamin intake and a high salt diet. A diet high in vegetables and fruits, citrus fruits, and fiber has been linked with lower risk of gastric cancer. Epigenetic fluctuation plays crucial roles in the initiation and progression of human gastric cancers. Gastric cancer is mostly asymptomatic with only non-specific symptoms in its initial stages (3,4). Consequently, by the time symptoms occur, the tumor has usually metastasized to other parts of the body. Regarding treatment of gastric cancer, surgery is still the best and last line of treatment for gastric cancer. Radiation therapy and chemotherapy as alternatives for surgery in the treatment of gastric cancer are not very promising. Despite the significant development of new surgical techniques, radiotherapy, chemotherapy, and targeted therapy, failures in gastric cancer treatment are still the most important challenges in oncology (5,6). The prognosis of gastric cancer is very poor, however, if detected at an early stage, long-term survival of gastric cancer is highly possible (7). As such there is an urgent need for the design and development of novel chemotherapeutic agents for the treatment of gastric cancer.

Among the new treatment regimens proposed for gastric cancer, is the use of complementary and alternative medicines. Numerous plant-derived substances, and their derivatives, are effective antitumor and chemopreventive agents. Naturally

Correspondence to: Dr Xi-Lin Song, Department of Gastrointestinal Surgery, Shandong Tumor Hospital and Institute, 440 Ji Yan Road, Jinan, Shandong 250017 P.R. China
E-mail: songxilin@hotmail.com

Key words: gastric cancer, apoptosis, 3- β -erythrodiol, cell cycle arrest

occurring plant derived molecules/extracts constitute a promising group of anticancer agents and have always played crucial roles for the treatment of numerous human cancers. In fact, more than 75% of the total number of commercially offered and clinically approved anticancer agents are either natural plant products or their semisynthetic derivatives. The well-known examples of plant based anticancer drugs are paclitaxel, vinblastine, vincristine, and camptothecin (8-11).

The aim of the present investigation was to assess the *in vitro* and *in vivo* anticancer and apoptotic activities of 3- β -erythrodiol isolated from *Conyza canadensis* in MKN-45 gastric cancer cells and a mouse xenograft model. Effects of 3- β -erythrodiol on cell cycle arrest, ROS generation and DNA fragmentation were also evaluated. No previous reports on the anticancer and apoptotic activities of 3- β -erythrodiol against MKN-45 gastric cancer cells have been reported, thus, the present study constitutes the first such report.

Materials and methods

Chemicals and reagents. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin, phosphate-buffered saline (PBS) with calcium chloride and magnesium chloride were obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). All other chemicals and solvents used were of the highest purity grade.

Plant material. The roots of *Conyza canadensis* were collected in May 2014 from Jinan City Shandong Province, China and identified by Professor Heng Song, a voucher specimen (voucher specimen no. 24-777-024-14) was deposited in the Herbarium of Southeast University (Nanjing, China).

Extraction, isolation and spectral data analysis. The air dried, finely powdered root material (2 kg) was extracted for 72 h with ethyl acetate in a soxhlet apparatus to afford the extract, which was concentrated under reduced pressure. The ethyl acetate extract (45 g) was charged on silica gel (60-120 mesh, 200 g) column and eluted with an increasing gradient of petroleum ether and ethyl acetate. Fractions of 50 ml volume each were collected and pooled according to TLC analysis. Five major fractions were collected (19:1, 9:1, 4:1, 1:1, 1:4). The fraction (petroleum ether:ethyl acetate, 19:1) yielded 3- β -erythrodiol as colorless crystalline solid, m.p. 229°C. The compound in its EIMS gave the molecular ion peak at m/z 442.3786 (calculated for $C_{30}H_{50}O_2$, 442.3810). The IR spectrum displayed absorption at 3,580, 3,430 cm^{-1} due to hydroxyl group and 1,610 cm^{-1} due to double bond. The 1H NMR spectrum displayed resonance signals due to seven tertiary methyl groups at δ 1.15, 0.98, 0.93, 0.92, 0.87, 0.86, 0.78, besides, a resonance signal for an olefinic proton at δ 5.70 (1H , t, $J=3.6$ Hz, H-12). The 1H NMR spectrum of the compound displayed the resonance signal due to a carbinylic proton at δ 3.12 (1H , d, $J_{ax-ax}=11.2$ Hz, $J_{ax-eq}=4.5$ Hz, H-3 α) indicating that the hydroxyl group at C-3 was equatorially oriented. The ^{13}C NMR spectral data of the compound indicated resonance for thirty carbons, resolved as, seven methyls, ten methylenes, five methines and seven

quaternary carbons. EIMS m/z : 442 (M)⁺, 409, 234, 216, 207, 204, 203, 189, 107, 95, 69. IR (KBr) ν max cm^{-1} : 3,580, 3,430 (O-H), 1,610 (C=C). 1H NMR ($CDCl_3$, 500 MHz) δ : 5.70 (1H , t, $J=3.6$ Hz, H-12), 3.12 (1H , d, $J_{ax-ax}=11.2$ Hz, $J_{ax-eq}=4.5$ Hz, H-3), 1.15 (3H , s, H-27), 0.98 (3H , s, H-23), 0.93 (3H , s, H-26), 0.92 (3H , s, H-25), 0.87 (3H , s, H-30), 0.86 (3H , s, H-29), 0.78 (3H , s, H-24). ^{13}C NMR ($CDCl_3$, 125 MHz) δ : 38.6 (C-1), 27.2 (C-2), 79.0 (C-3), 38.8 (C-4), 55.1 (C-5), 18.4 (C-6), 32.6 (C-7), 39.8 (C-8), 47.6 (C-9), 36.9 (C-10), 23.6 (C-11), 122.4 (C-12), 144.2 (C-13), 41.8 (C-14), 25.6 (C-15), 22.0 (C-16), 36.9 (C-17), 42.3 (C-18), 46.5 (C-19), 30.9 (C-20), 34.1 (C-21), 31.0 (C-22), 28.1 (C-23), 15.6 (C-24), 15.5 (C-25), 16.6 (C-26), 25.9 (C-27), 69.5 (C-28), 33.2 (C-29), 23.11 (C-30). Comparison of physical characteristics and spectral data with that reported in literature (12), confirmed it to be 3- β -erythrodiol.

Cell line and culture conditions. The MKN-45 human gastric cancer cell line was purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). These cells were cultured in DMEM supplemented with 10% (v/v) FBS (Hyclone, USA) under humidified atmosphere of 5% CO_2 at 37°C. The medium was replaced every 2 days. Cells were subcultured every 4 days.

MTT assay for cell proliferation. The effects of 3- β -erythrodiol on MKN-45 cell viability were examined by MTT assay. Cells (2×10^5 cells/well in 100 μ l medium) were seeded into 96-well plates for 24 h before drug treatment. Subsequent drug treatment with various doses of 3- β -erythrodiol (0, 2.5, 5, 25, 50, 75 and 100 μ M) for 24 and 48 h, the cell plates were then treated with MTT solution (10 μ l; 5 mg/ml in PBS solution) for an additional 2 h at 37°C. The formazan crystals in viable cells were solubilized with DMSO (150 μ l) and the absorbance was measured on a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 490 nm. The effects of 3- β -erythrodiol on cell viability were calculated as an inhibition ratio (1%) using the following equation (OD_{490} , optical density at 490 nm):

$$I\% = \frac{[OD_{490}(\text{Control}) - OD_{490}(\text{Treated})]}{OD_{490}(\text{Control})} \times 100\%$$

Evaluation of LDH release. MKN-45 human gastric cancer cells were seeded into a 96-well plate and treated with 3- β -erythrodiol for 48 h, then 20 μ l LDH was added to release the reagent. After 1 h, the cell culture plate was centrifuged at 500 g for 15 min and 150 μ l supernatant from each well was collected into a new black 96-well plate. Next, 100 μ l of LDH assay mixture was added to each well, and the plate was incubated at 37°C for 20 min. The absorbance was measured spectrophotometrically at 490 nm wavelength.

Phase contrast and fluorescence microscopic study of gastric cancer cell morphology. MKN-45 gastric cancer cells were seeded into 6-well plates at a density of 2×10^5 cells/well in 20 ml medium. The cells were treated with varying concentrations (0, 5, 50 and 100 μ M) of 3- β -erythrodiol for 48 h. The morphological alterations were observed and the images were captured under an inverted light microscope (Nikon Corporation, Tokyo, Japan) after 48 h. The same spot of

cells was noted and captured. The images were captured at a magnification of x200.

The fluorescence microscopic staining images were recorded using a UV fluorescence microscope (Olympus; Olympus Optical Co., Ltd., Tokyo, Japan) using UV filter at x200 magnification to detect morphological evidence of apoptosis. In brief, MKN-45 human gastric cancer cells were seeded into 12-well plates at a density of 2×10^5 cells/well and then treated with 0, 5, 50 and 100 μ M dose of 3- β -erythrodiol for 48 h. The apoptotic changes were evaluated by Hoechst staining kit according to the instructions of the manufacturer. After drug treatment, the cells were fixed with 5% polyoxymethylene and then incubated in Hoechst solution for 10-15 min in the dark. The images were then captured by the fluorescence microscope.

Propidium iodide and acridine orange double staining assay for apoptosis quantification. The apoptotic cell death induced by 3- β -erythrodiol in MKN-45 human gastric cancer cells was quantified by using propidium iodide (PI) and acridine orange (AO) double staining as per the manufacturer guidelines and the cells were observed under fluorescence microscope (Olympus; Olympus Optical Co., Ltd.). Briefly, MKN-45 cells were plated at a density of 1×10^5 cells/ml, and treated with 0, 5, 50 and 100 μ M of 3- β -erythrodiol for 48 h. The cells were incubated in 5% CO₂ atmosphere at 37°C. The cells were then centrifuged at 12,000 rpm for 10 min, supernatant was discarded and the cells were washed twice using PBS. Ten microliters each of propidium iodide and acridine orange were added into the cell pellet. Freshly stained cell suspension was dropped into a glass slide and covered by a coverslip. The glass slides were examined under a UV-fluorescence microscope.

Scanning electron microscopy (SEM) studies of exterior ultra-structural cellular features. MKN-45 human gastric cancer cells at a density of 2×10^6 cells were seeded in 6-well microtitre plates. Various doses of 3- β -erythrodiol were added to the cell culture followed by incubation of 12 h. The cells were centrifuged at 10,000 rpm for 10 min followed by PBS washing. The supernatant was removed and resuspended in 10 ml of 0.1 M cacodylate buffer. The sample was again centrifuged at 10,000 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 5 ml of 3.2% glutaraldehyde in 0.1 M cacodylate buffer. The cacodylate buffer was removed and 1.2% of osmium tetroxide (OsO₄) in 0.2 M cacodylate buffer was added. The cover slips were washed three times with 0.2 M cacodylate buffer. Then 30% ethanol for 5 min was added. The coverslips were glued onto stubs with silver print and dried for about 20 min at room temperature. Then the samples were covered with gold using sputter coater. The images were captured by a scanning electron microscope (JOEL 64000, Japan) at accelerating voltage of 15-25 KV).

Measurement of intracellular ROS generation. Intracellular ROS generation was evaluated using fluorescent CM-DCFH2-DA. MKN-45 cells were seeded in 6-well plates and after adhesion, the cells were pretreated with 10 μ M CM-DCFH2-DA for 30 min followed by co-incubation with various concentrations of 3- β -erythrodiol for another 3 h and washed with ice-cold PBS twice. The cells were collected

and analyzed using a flow cytometry (FACSCanto™; Becton Dickinson, Franklin Lakes, NJ, USA) with wavelength of excitation and emission at 488 and 525 nm, respectively.

DNA fragmentation analysis by gel electrophoresis. MKN-45 human gastric cancer cells were seeded in a 100-mm cell culture dish for 24 h, and treated with 0, 5, 50 and 100 μ M 3- β -erythrodiol for 48 h. The cells were harvested and washed with PBS, and the pellets were lysed with a 400- μ l DNA lysis buffer (2% NP-40, 20 mM EDTA, 40 mM Tris-HCl) for 30 min. After centrifugation, the supernatants were prepared in an equal volume of 1.5% sodium-dodecyl sulphate, incubated with 2.5 mg/ml RNase A at 60°C for 2 h followed by digestion with 2.5 mg/ml proteinase K for 2 h at 20°C. Following the addition of 0.5 volumes of 10 M ammonium acetate, the DNA was precipitated with cold ethanol and collected by centrifugation at 15,000 rpm for 30 min. DNA was then dissolved in gel loading buffer, separated by electrophoresis in 1.5% agarose gel and visualized under UV light, following ethidium bromide staining.

Effect of 3- β -erythrodiol on cell cycle progression. Effect of 3- β -erythrodiol on cell cycle was analyzed by flow cytometry using a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA), equipped with CellQuest 3.3 software. ModFit LT cell cycle analysis software was used to determine the percentage of cells in the various phases of the cell cycle. Briefly, MKN-45 human gastric cancer cells (1×10^5 cells) were treated with numerous doses of 3- β -erythrodiol (0, 5, 50 and 100 μ M) for 48 h. Subsequently, the cells were collected, washed with ice cold PBS, fixed with 70% alcohol at 4°C for 12 h and stained with propidium iodide in the presence of 3% RNAase A at 37°C for 20 min prior to analysis using flow cytometry.

In vivo experiments. The effects of 3- β -erythrodiol on tumor development were examined using a nude mouse model. Male Balb/c nude mice (6 weeks old) were purchased from SLAC Laboratory Animal Co. (Shanghai, China), and all mice were maintained with water and food *ad libitum* in a pathogen free environment with a 12-h light and 12-h dark cycle in an animal care facility and according to Animal Welfare regulations and protocols approved by the Institutional Animal Care and Use Committee of Shandong Provincial Hospital (Jinan, Shandong, China). The MKN-45 human gastric cancer cells (1×10^5 cells/mouse) were subcutaneously injected into the right rear flank of each mouse (5-6 mice/group) to produce tumors in mice. After tumor development, the mice were divided into 3 groups and treated with 3- β -erythrodiol injected intraperitoneally. The control group in the study was treated with an equal amount of PBS. Afterwards, the mice were sacrificed after 24 days and the tumor weight and volume of each mouse were evaluated. Tumor length and width were measured using a caliper and the tumor volume was calculated using the formula: tumor volume = length x width x 0.5 width.

Statistical analysis. The results indicate values from three independent experiments with the data expressed as the means \pm SD. Differences between the control and treatment groups were examined using the Student's t-test with SPSS 17.0

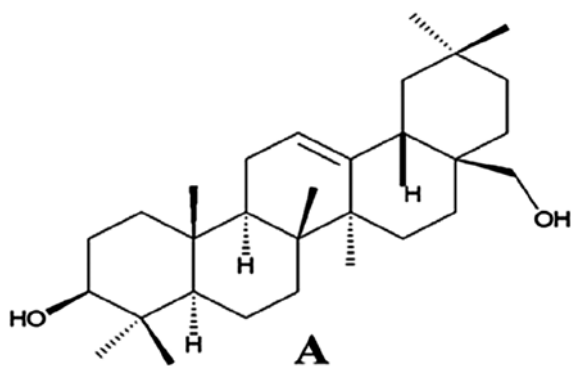


Figure 1. Chemical structure (A), ^1H -NMR and ^{13}C -NMR of 3- β -erythrodiol.

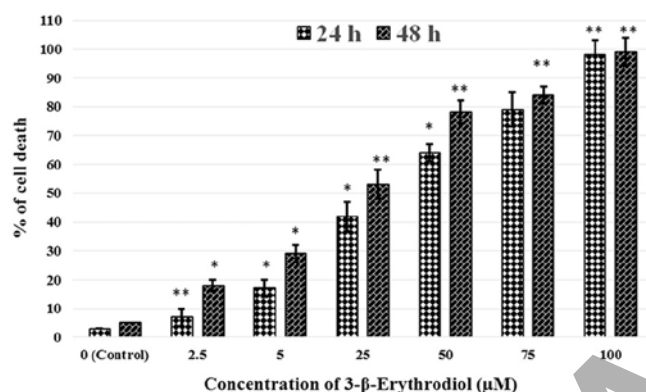


Figure 2. Cytotoxic effect of 3- β -erythrodiol in human gastric cancer cells (MKN-45). Data are shown as the mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, vs. 0 μM (control).

software. A p -value < 0.05 was considered to indicate a statistically significant difference.

Results

Effects of 3- β -erythrodiol on the cytotoxicity of human gastric cancer cells (MKN-45). The structure of 3- β -erythrodiol is shown in Fig. 1. The cytotoxic effects of the 3- β -erythrodiol in human gastric cancer cells were evaluated by MTT as well as LDH release assay. Initially we demonstrated the anticancer activity of 3- β -erythrodiol on MKN-45 cells by using MTT assay. The results revealed that 3- β -erythrodiol exerted potent anti-proliferative effects on these cancer cells. It showed both concentration-dependent as well as time-dependent growth inhibitory effects against these cells (Fig. 2). For determining the effectiveness of this triterpene compound, its IC_{50} value was also calculated to be 45.2 and 21.6 μM at 24 and 48 h, respectively. In addition, the lactate dehydrogenase (LDH) released to the culture medium was also increased in a concentration-dependent manner (Fig. 3). These two assays indicated that 3- β -erythrodiol induced potent cytotoxic effects in MKN-45 cells in a dose-dependent manner.

Morphological study of MKN-45 human gastric cancer cells using phase contrast and fluorescence microscopy. In this study, the morphological alterations of human gastric cancer cells (MKN-45) untreated and treated with 3- β -erythrodiol

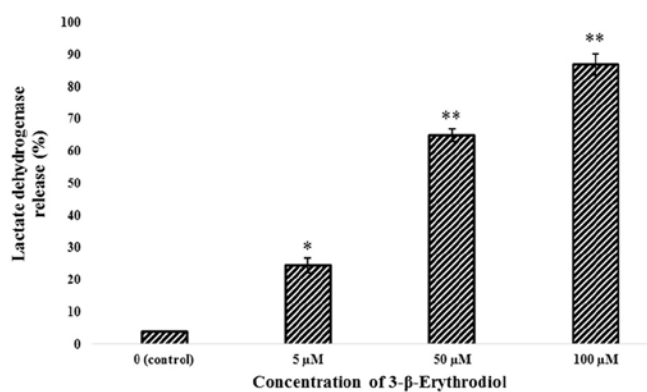


Figure 3. MKN-45 human gastric cancer cells were treated with various concentrations of 3- β -erythrodiol for 48 h, LDH release was detected by LDH assay kit. Each point is the mean \pm SEM of three experiments (* $P < 0.05$; ** $P < 0.01$ vs. control group).

were identified under a phase contrast microscope. In comparison to the control-treated cells, the cells treated with 5, 50 and 100 μM of 3- β -erythrodiol exhibited a significant reduction in cell viability (Fig. 4). Untreated MKN-45 cells were tightly packed and organized multilayers, whereas after incubation with various concentrations of 3- β -erythrodiol for 48 h numerous cells became rounded and withered, and disconnected from each other or floated in the medium.

The process of apoptosis is characterized by certain morphological changes including reduction in cell volume, and chromatin condensation in the nucleus. To confirm whether 3- β -erythrodiol induces apoptosis in MKN-45 human gastric cancer cells, fluorescence microscopy using Hoechst 33342 as a staining agent was used. Following treatment with 5, 50 and 100 μM dose of 3- β -erythrodiol in MKN-45 cells, the most prominent morphological changes were observed in the treated cells as compared to the untreated cells. As shown by phase contrast microscopy (Fig. 4), the untreated control cells were morphologically normal. Reduction in the cell population and change in cellular morphology were observed with 3- β -erythrodiol treatment. Higher doses of 3- β -erythrodiol induced chromatin condensation, fragmented nuclei and nuclear shrinkage.

3- β -Erythrodiol induced early and late apoptosis (propidium iodide and acridine orange double staining assay). Apoptotic, necrotic, and living MKN-45 gastric cancer cells were counted under a fluorescence microscope. Around 400 cells were randomly and differentially counted and it was observed that 3- β -erythrodiol initiated and induced apoptotic morphological features in a concentration-dependent manner (Fig. 6). Untreated cells (Fig. 6A) were shown to have green color showing normal nuclear structure. Early apoptosis is detected by intercalation of acridine orange within the fragmented DNA, which is indicated by fluorescent bright-green color (Fig. 6B). At higher doses of 3- β -erythrodiol, nuclear margination and blebbing was detected while as at very high doses of 3- β -erythrodiol, late stages of apoptosis were observed with appearance of apoptotic body formation and reddish-orange fluorescence (Fig. 6C and D). Differential counting of treated MKN-45 cells indicated that there is a statistically significant

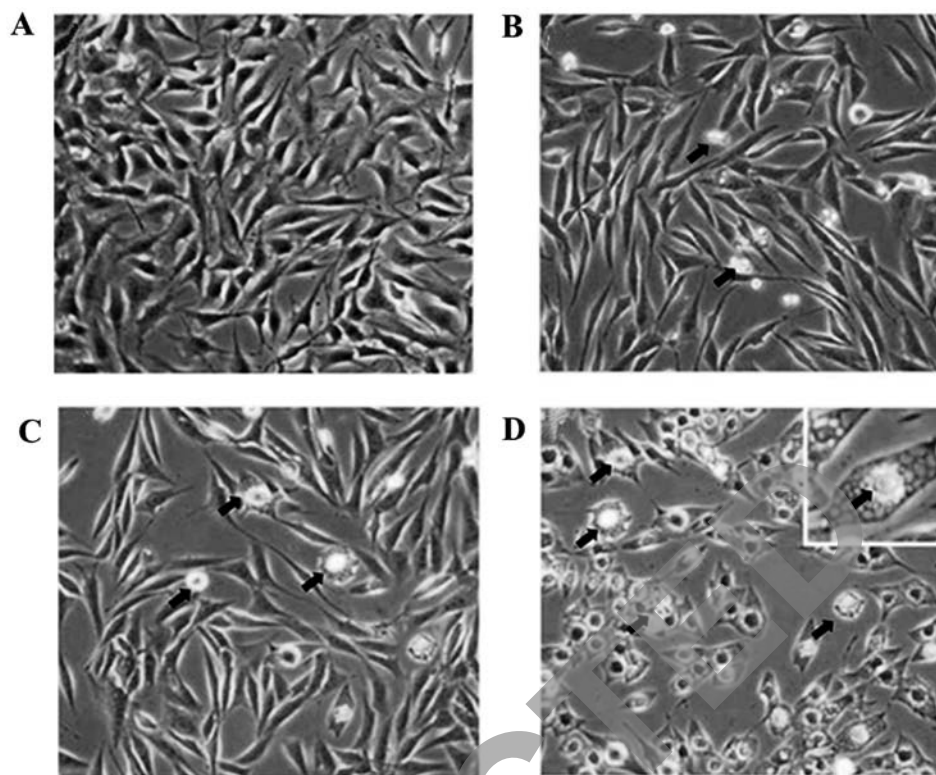


Figure 4. Morphological changes of the cells after treatment with 3- β -erythrodiol (magnification x200) detected by phase contrast microscopy. Cellular shrinkage was observed in 3- β -erythrodiol-treated cells (black arrows). (A) Control (untreated cells), (B-D) effect of 5, 50 and 100 μ M of 3- β -erythrodiol on cell morphology of MKN-45 cells. The number of cells with shrinkage increased with the increasing dose of the compound.

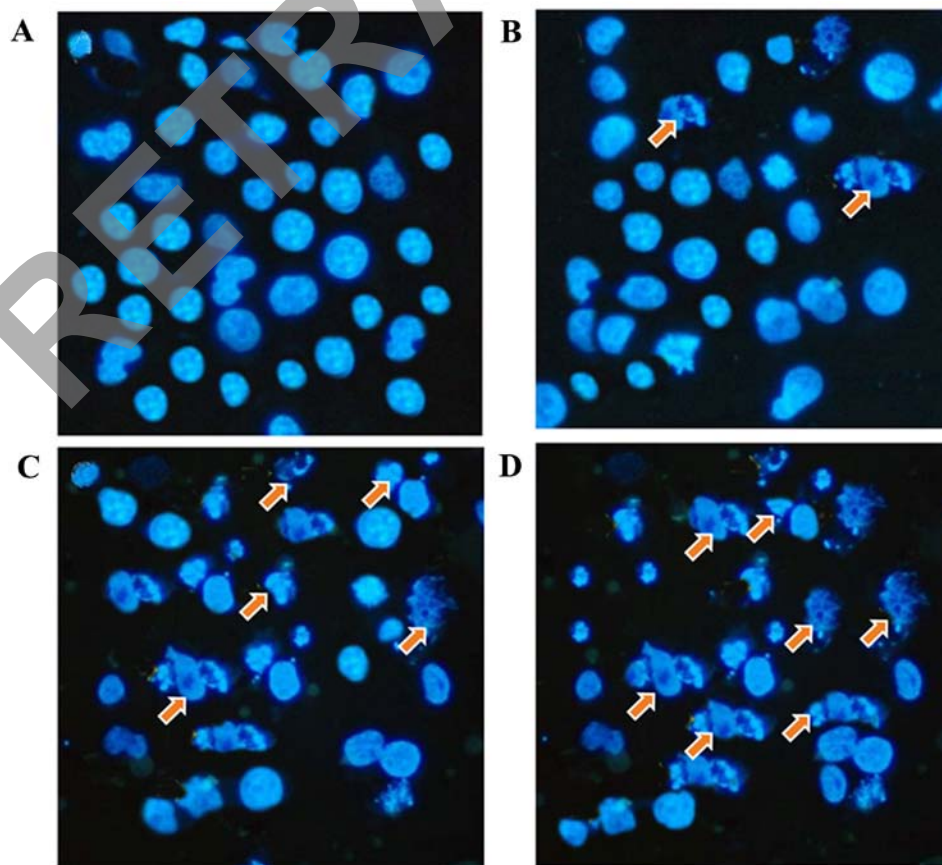


Figure 5. Morphological features of apoptosis including chromatin condensation induced by 3- β -erythrodiol (0 μ M, A), (5 μ M, B), (50 μ M, C), (100 μ M, D) for 48 h. (A) represents untreated control group which does not show any changes in cellular morphology. Arrows represent morphological changes in the gastric cancer cells. Hoechst 33342 was used as a staining agent and fluorescence microscopy was used to capture the images.

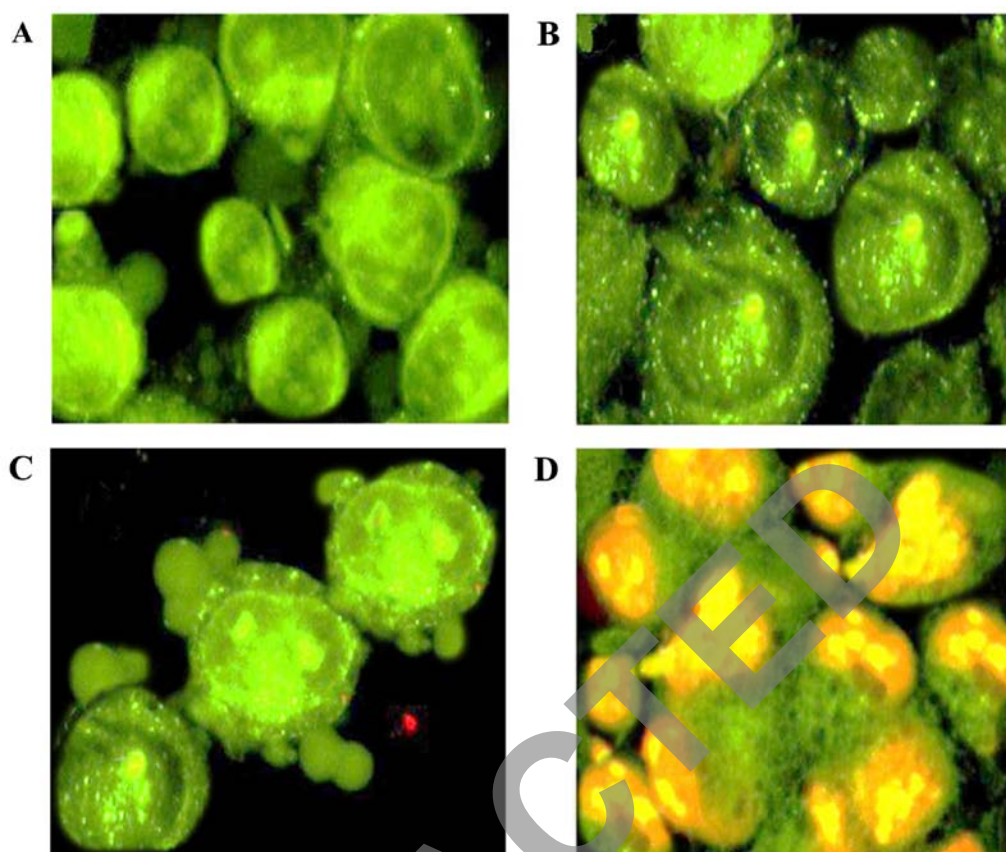


Figure 6. The fluorescent micrographs (magnification x400) of MKN-45 human gastric cancer cells double-stained with acridine orange and propidium iodide. Untreated cells (A) reveal normal cell morphology without any signs of apoptosis while (B, 5 μ M) shows signs of early apoptosis, (C, 50 μ M) shows nuclear margination and blebbing and (D, 100 μ M) shows signs of late apoptosis.

($P < 0.05$) difference in apoptosis positive cells, which indicates clearly that 3- β -erythrodiol has a concentration-dependent apoptogenic effect. The percentage of apoptotic cells increased from 5.2% in control cells to 26.5, 43.2 and 74.3% in 5, 50 and 100 μ M 3- β -erythrodiol-treated cells, respectively.

MKN-45 cell surface analysis by scanning electron microscopy (SEM). SEM was carried out indicating the cell surface morphology. The procedure of SEM in the analysis of apoptosis is primarily referred to the study of cell surface alterations such as smoothing, loss of microvillus structures, blebbing, and shrinking. As shown in Fig. 7A, SEM examination reveals that untreated MKN-45 human gastric cancer cells are spherical in shape with smooth surface. However as shown in Fig. 7B, 3- β -erythrodiol treatment to MKN-45 cells at 50 μ M resulted in few surface projections and blebbing of the plasma membrane. Furthermore, when the concentration of the 3- β -erythrodiol was enhanced to 100 μ M, a complete apoptotic body formation was observed. Overall, the SEM data clearly demonstrated that the atypical apoptotic phenomena occurred in MKN-45 cells treated with 3- β -erythrodiol. However, the relative appearance of apoptotic features were more evident at higher doses.

3- β -Erythrodiol induces ROS formation in MKN-45 human gastric cancer cells. The effect of 3- β -erythrodiol on intracellular ROS production was measured by flow cytometry with a fluorescent probe CM-DCFH2-DA. As shown in Fig. 8,

after treating MKN-45 cells with 3- β -erythrodiol for 3 h, it profoundly induced ROS formation. A concentration dependent ROS generation was witnessed and 3-fold increase of ROS production was seen after 100 μ M 3- β -erythrodiol treatment.

3- β -Erythrodiol induces DNA fragmentation in MKN-45 gastric cancer cells. In addition to the morphological changes of apoptosis in 3- β -erythrodiol-treated MKN-45 cells, DNA fragmentation was also studied by observation of the formation of DNA ladder. As shown in Fig. 9, DNA ladder seemed to be more marked with the increasing 3- β -erythrodiol concentration, however, no DNA fragments were observed in the control groups (Fig. 9, 0 μ M). However, 5, 50 and 100 μ M doses of the 3- β -erythrodiol after 48 h exposure led to a substantial increase in DNA fragmentation (Fig. 9, right panel). The DNA fragmentation is a hallmark of apoptosis, further confirming that the 3- β -erythrodiol induced cell death via apoptosis.

Effect of 3- β -erythrodiol on cell cycle phase distribution in MKN-45 human gastric cancer cells. Apoptosis and cell cycle are intimately connected biochemical processes, and any disruption in cell cycle progression may eventually result in apoptotic cell death. With the purpose of having a mechanistic indication of the growth inhibitory effect exerted by 3- β -erythrodiol in MKN-45 cancer cells, flow cytometry analysis was performed to identify whether the compound induces cell cycle arrest in this cell line. The results indicated that 3- β -erythrodiol induces sub-G1 cell cycle arrest

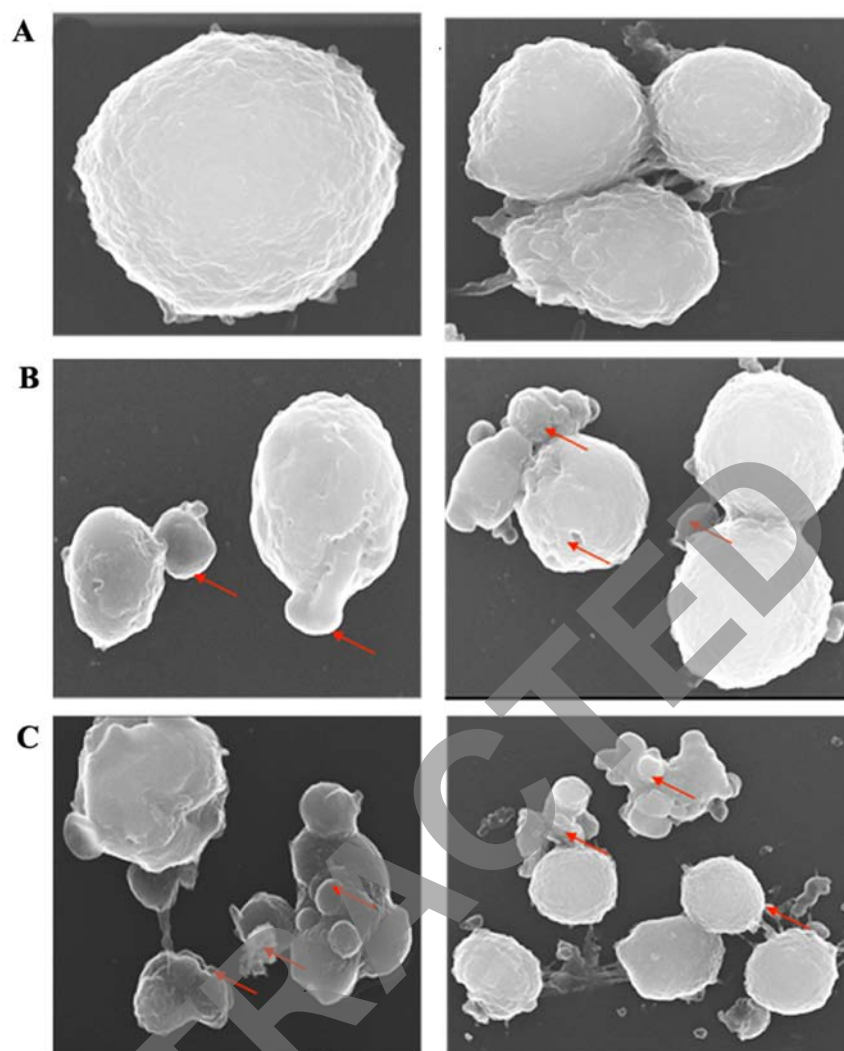


Figure 7. Scanning electron microscopy in MKN-45 human gastric cancer cells. (A) SEM of untreated MKN-45 cells, (B) MKN-45 cells treated with 3- β -erythrodiol at 50 μ M for 48 h and (C) MKN-45 cells treated with 3- β -erythrodiol at 100 μ M. The untreated control cells exhibited rough surface and microvilli. The treatment for 48 h caused reduction in cell size, smoothening of cell surface, blebbing of the plasma membrane (B) and treatment at 100 μ M for 48 h, transformed the cells into apoptotic bodies. (A-C) In each case describes magnification at x2,000, x4,000 and x6,000, respectively. Data are representative of three independent experiments.

and increases the portion of apoptotic cells. To determine the distribution of 3- β -erythrodiol-treated MKN-45 cells in different phases of the cell cycle, DNA content in cells was detected by propidium iodide (PI) staining and flow cytometry. Treatment with different concentrations of the compound for 48 h led to an increase in the population of cells in the sub-G0/G1 phase (apoptotic population) ($P < 0.05$) (Fig. 10). This increase in sub-G1 population was accompanied by a corresponding reduction of the cells in S-phase and an increase in G2/M phase of the cell cycle. As compared to the control (Fig. 10A), 3- β -erythrodiol-treated (5 μ M Fig. 10B, 50 μ M C, and 100 μ M D) cells showed a significant proportion of cells in apoptosis (sub-G1).

3- β -Erythrodiol reduces tumor volume and tumor weight in male Balb/c nude mice. *In vitro* findings reveal that 3- β -erythrodiol is a potent cytotoxic agent inhibiting cell proliferation and inducing apoptosis and cell cycle arrest. Next step was to demonstrate whether this compound induces the same anticancer effects also *in vivo*. Cancer was induced in the

mice by injecting MKN-45 cancer cells (1×10^6 cells/mouse). Subsequent to the tumor development, the mice were sacrificed and tumors were removed and their weights and volumes were measured (Fig. 11). The findings revealed that 0.50 and 1.0 μ g/g 3- β -erythrodiol injection decreased the tumor weight from 1.40 g in PBS-treated group (control) to 0.61 and 0.22 g, respectively (Fig. 11A and B). Similarly, 0.50 and 1.0 μ g/g 3- β -erythrodiol injection reduced the tumor volume from 1.5 cm^3 in PBS-treated group (control) to 0.91 and 0.31 cm^3 , respectively (Fig. 11A and C).

Discussion

Conyza canadensis, commonly referred to as Canada fleabane. The other names include horseweed, butterweed, Canadian horseweed (13). It is an annual plant native of Northern and Central America, but has spread over almost all parts of the world. *Conyza canadensis* is an annual plant growing to 1.5 m tall, with sparse hairy stems. The leaves are slender, 2-10 cm long and up to 1 cm broad, with a coarse toothed margin. The

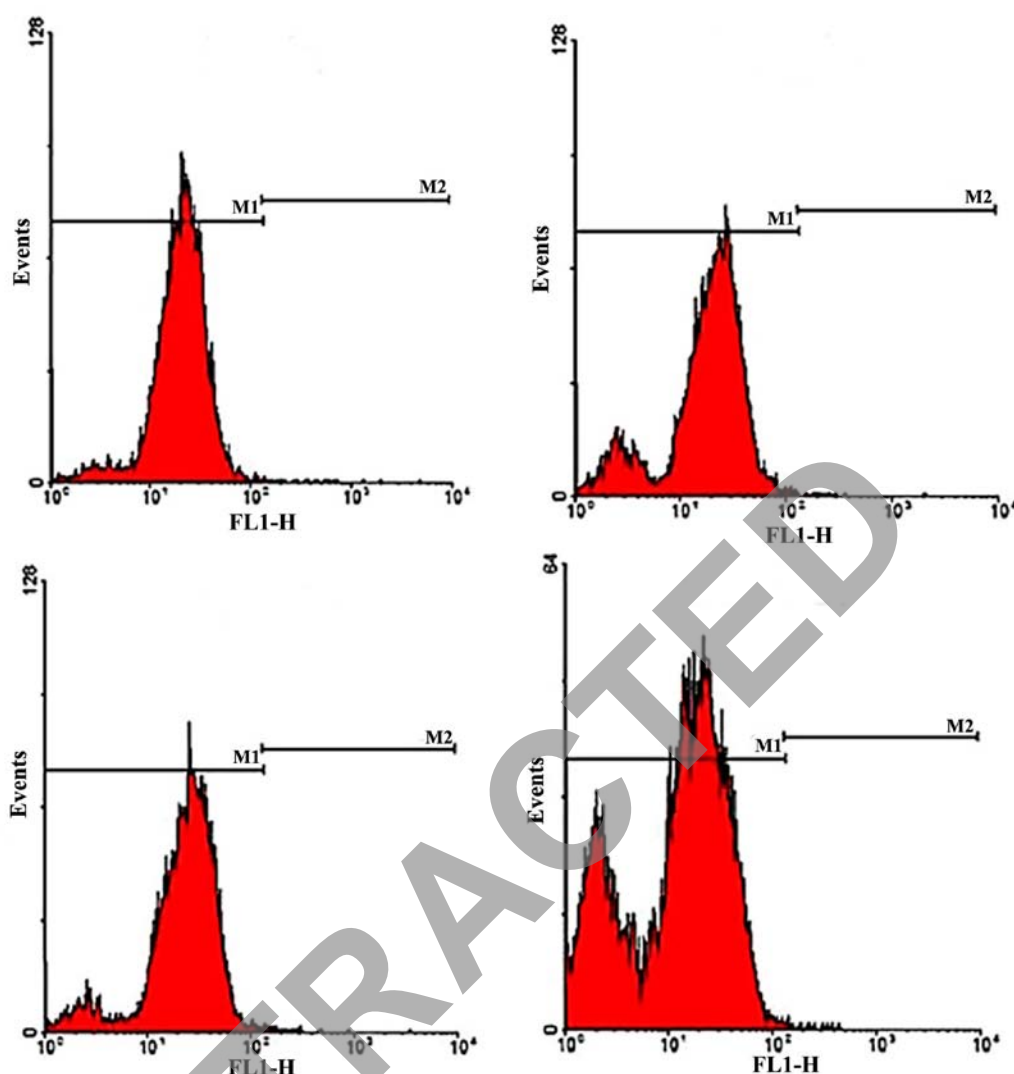


Figure 8. Effect of 3- β -erythrodiol on production of reactive oxygen species (ROS). Histogram representing the effect of 3- β -erythrodiol on ROS production in MKN-45 human gastric cancer cells. Cells were treated with 3- β -erythrodiol (0, 5, 50 and 100 μ M) and harvested after 2 h of treatment. Cells were stained with DCFDA and subjected to flow cytometric analysis.

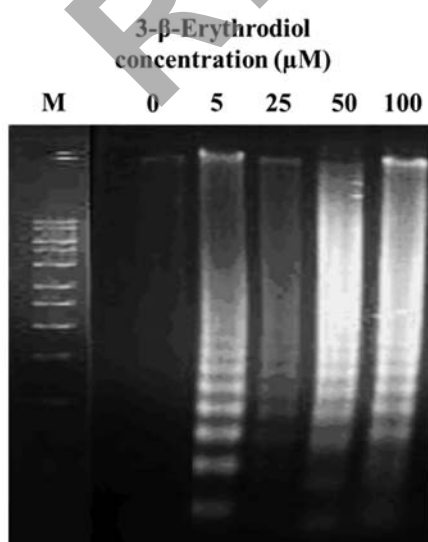


Figure 9. 3- β -Erythrodiol induces DNA fragmentation in human gastric cancer cells (MKN-45). The cells were treated with 0, 5, 25, 50 and 100 μ M of 3- β -erythrodiol for 48 h. Cells from each sample were harvested for DNA gel electrophoresis as described in Materials and methods.

aerial parts of *Conyza canadensis* have been used in different parts of the world to treat several ailments, most commonly diarrhoea and dysentery, and as a diuretic agent. The petroleum ether and ethanolic extracts of aerial parts exhibit significant anti-inflammatory activity (14). In Chinese folk medicine, *Conyza canadensis* has also been applied for the treatment of wounds, swellings, and pain caused by arthritis (15). The whole plant is locally used for the treatment of edema, hematuria, hepatitis and cholecystitis (16). Moreover, a decoction of horseweed has traditionally been used to treat cancerous diseases in North America (17). Numerous species of genus *Conyza* have been reported to be rich sources of diterpenes (18,19). Earlier phytochemical studies of *C. canadensis* revealed the presence of triterpenoids, sterols, sphingolipids (20,21), specific C-10 acetylenes 46-47, sesquiterpene hydrocarbons (22) and flavonoids (23). Recently, a triterpenoid ester, 3- β , 16- β , 20- β -trihydroxytaraxa-stane-3-O-palmitoyl ester, and phenylpropanoyl 2,7-anhydro-3-deoxy-2-octulosonic acid derivatives (24) were isolated. Phytochemical analysis of the essential oil of *C. canadensis* revealed constituents, including

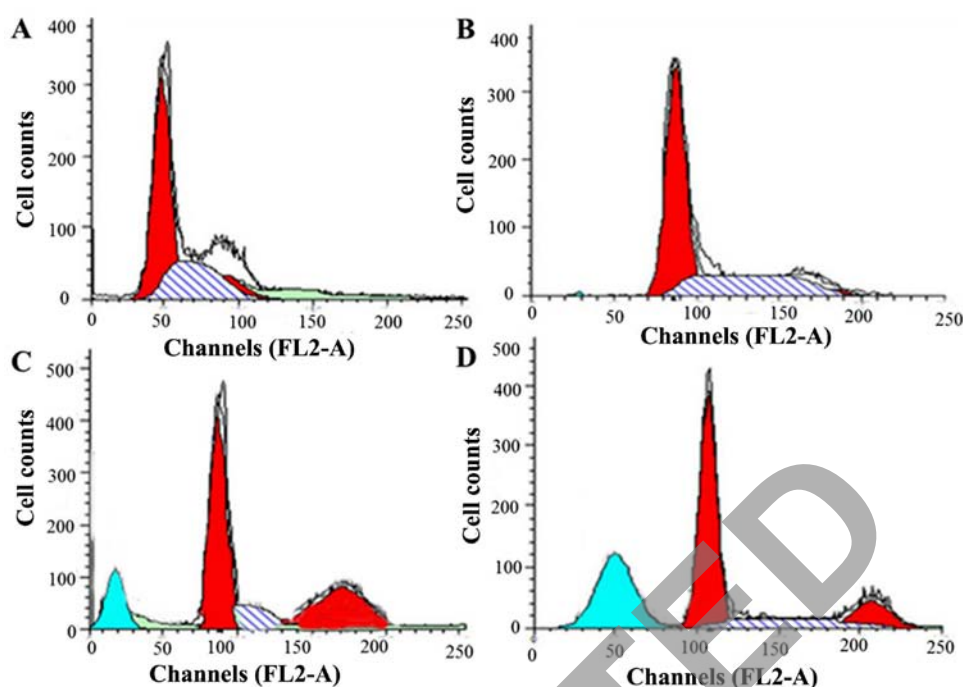


Figure 10. 3- β -Erythrodiol induced cell cycle arrest at sub-G1 phase in human gastric cancer cells (MKN-45). The cells were subjected to 0 μ M (untreated, A), 5 μ M (B), 50 μ M (C) and 100 μ M (D) of 3- β -Erythrodiol. The cells in the sub-G1 phase (apoptotic cells) increased from A-D, with an increase in 3- β -erythrodiol dose. The DNA histogram shows the distribution and the percentage of cells in different phases of the cell cycle.

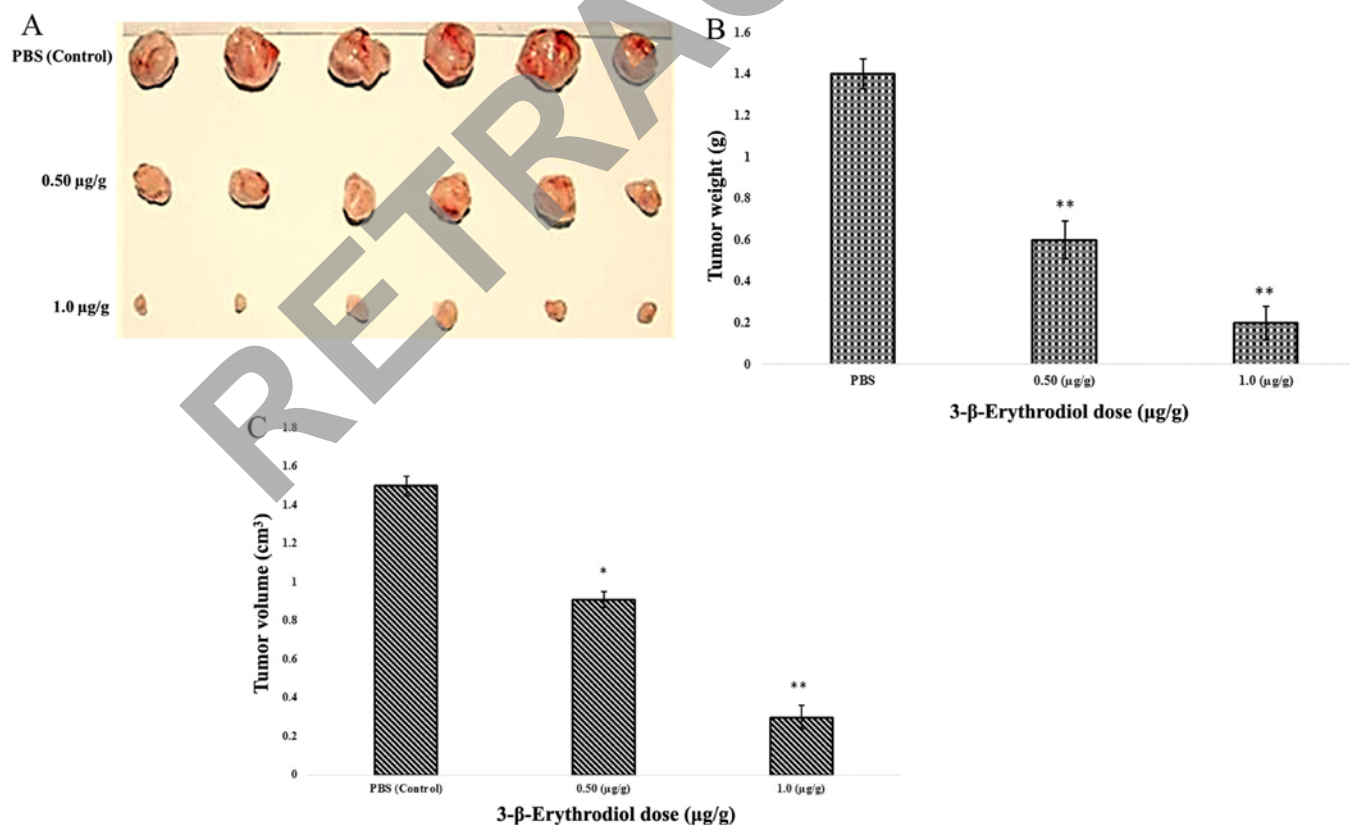


Figure 11. (A) 3- β -Erythrodiol inhibits tumor growth in a mouse xenograft model. Images of cancerous tissues removed from the mice in control and 3- β -erythrodiol-treated groups. (B) 3- β -Erythrodiol reduces tumor weight in the treated mice. The values are the means \pm SEM (n=6). **P<0.01. (C) 3- β -Erythrodiol reduces tumor volume in the treated mice. The values are the means \pm SEM. **P<0.01, *P<0.05.

monoterpenes, sesquiterpenes, and acetylenes, among which d-limonene was the predominant constituent (25).

In this investigation, our aim was to evaluate the *in vitro* and *in vivo* anticancer and apoptotic effects of 3- β -erythrodiol

against MKN-45 human gastric cancer cells and in a mouse xenograft model. 3- β -Erythrodiol was initially isolated from the ethyl acetate root extract of *Conyza canadensis* by column chromatography and then its structure was evaluated by different spectral techniques.

Many plant based compounds have been reported to curb cancer cell growth which paves the way for anticancer drug discovery. It has also been reported that reactive oxygen species (ROS)-mediated DNA damage after treatment with plant based chemotherapeutic agents is an essential contributing factor in the induction of apoptosis and cell death (26). Therefore, agents that can cause DNA damage and subsequent apoptosis will act as potent anticancer drugs to control the process of tumorigenesis and tumor recurrence (27,28). Furthermore, development of good leads for drug discovery should selectively be able to induce apoptosis in cancer cells (29) without causing excessive damage to normal cells. Apoptosis may be useful in the management and therapy of cancer. Apoptosis gives clues on effective anticancer therapy and many chemotherapeutic agents have been reported from the search of herbal and other natural products influencing apoptosis (30).

The current investigation revealed that 3- β -erythrodiol induced apoptosis in MKN-45 human gastric cancer cells. At lower concentration of 3- β -erythrodiol, nuclear margination and blebbing was detected while at very high doses of 3- β -erythrodiol, late stages of apoptosis were observed with appearance of apoptotic body formation with reddish-orange fluorescence. Differential counting of treated MKN-45 cells indicated that there is a statistically significant ($P < 0.05$) difference in apoptosis positive cells, which indicates clearly that 3- β -erythrodiol has a concentration-dependent apoptogenic effect. The scanning electron microscopy data clearly demonstrated that the atypical apoptotic phenomena occurred in MKN-45 cells treated 3- β -erythrodiol with appearance of few surface projections and blebbing of the plasma membrane. After treating MKN-45 cells with 3- β -erythrodiol for 3 h, it strongly induced ROS formation. A dose-dependent ROS generation was witnessed and 3-fold increase of ROS production was seen after 100 μ M 3- β -erythrodiol treatment. DNA ladder seemed to be more evident with the increasing 3- β -erythrodiol concentration, however, no DNA fragments were observed in the control groups. The DNA fragmentation is a hallmark of apoptosis, further confirming that the 3- β -erythrodiol induced cell death via apoptosis. 3- β -Erythrodiol also induced sub-G1 cell cycle arrest in these cancer cells. Disruption in cell cycle progression may also result in apoptotic cell death because there is an intimate relation between cell cycle and apoptosis. 3- β -Erythrodiol has been earlier reported to exhibit anti-proliferative and pro-apoptotic activities in astroglial tumor cells (1321N1) by inducing sub-G0/G1 cell cycle arrest (31).

In vivo studies revealed that 3- β -erythrodiol injection decreased the tumor weight and volume in male Balb/c nude mice. It was observed that 0.50 and 1.0 μ g/g 3- β -erythrodiol injection decreased the tumor weight from 1.40 g in PBS-treated group (control) to 0.61 and 0.22 g, respectively, while the tumor volume was reduced from 1.5 cm³ in PBS-treated group (control) to 0.91 and 0.31 cm³ after treating with 0.50 and 1.0 μ g/g 3- β -erythrodiol injection, respectively.

In conclusion, the present investigation reveals that 3- β -erythrodiol isolated from *Conyza canadensis* exerts potent anti-proliferative effects in human gastric cancer by inducing early and late apoptosis, cell cycle arrest, and ROS generation. It also decreased the tumor volume and tumor weight in male Balb/c nude mice.

Acknowledgements

This study was supported by the Traditional Chinese Medicine Science and Technology Development Plan of Shandong Province (no. 2013-210).

References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 127: 2893-2917, 2010.
2. Yang L, Parkin DM, Ferlay J, Li L and Chen Y: Estimates of cancer incidence in China for 2000 and projections for 2005. *Cancer Epidemiol Biomarkers Prev* 14: 243-250, 2005.
3. Hwang H, Dwyer J and Russell RM: Diet, *Helicobacter pylori* infection, food preservation and gastric cancer risk: Are there new roles for preventative factors? *Nutr Rev* 52: 75-83, 1994.
4. Rasul A, Yu B, Yang LF, Ali M, Khan M, Ma T and Yang H: Induction of mitochondria-mediated apoptosis in human gastric adenocarcinoma SGC-7901 cells by kuraridin and Nor-kuraridin isolated from *Sophora flavescens*. *Asian Pac J Cancer Prev* 12: 2499-2504, 2011.
5. Baeza MR, Giannini TO, Rivera SR, González P, González J, Vergara E, del Castillo C, Madrid J and Vinés E: Adjuvant radiochemotherapy in the treatment of completely resected, locally advanced gastric cancer. *Int J Radiat Oncol Biol Phys* 50: 645-650, 2001.
6. Longley DB, Harkin DP and Johnston PG: 5-fluorouracil: Mechanisms of action and clinical strategies. *Nat Rev Cancer* 3: 330-338, 2003.
7. Jemal A, Center MM, DeSantis C and Ward EM: Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev* 19: 1893-1907, 2010.
8. Newman DJ, Cragg GM and Snader KM: The influence of natural products upon drug discovery. *Nat Prod Rep* 17: 215-234, 2000.
9. Newman DJ and Cragg GM: Natural products as sources of new drugs over the last 25 years. *J Nat Prod* 70: 461-477, 2007.
10. Oberlies NH and Kroll DJ: Camptothecin and taxol: Historic achievements in natural products research. *J Nat Prod* 67: 129-135, 2004.
11. van Der Heijden R, Jacobs DI, Snoeijer W, Hallard D and Verpoorte R: The *Catharanthus* alkaloids: Pharmacognosy and biotechnology. *Curr Med Chem* 11: 607-628, 2004.
12. Xue HZ, Lu ZZ, Konno C, Soejarto DD, Cordell GA, Fong HHS and Hodgson W: 3-Beta-(3,4-dihydroxycinnamoyl)-erythrodiol and 3-beta-(4-hydroxycinnamoyl)-erythrodiol from *Larrea tridentata*. *Phytochemistry* 27: 233-235, 1988.
13. Arnold C: In A guide to Medicinal Plants of United States. The New York Times Book Co. 93, 1980.
14. Khare CP: Indian Medicinal Plants. An illustrated Dictionary. Springer, p242, 2007.
15. Li TSC: Chinese and Related North American Herbs - Phytopharmacology and Therapeutic values. CRC Press, Boca Raton, London, New York, Washington, p179, 2002.
16. Ling Y, Chen Y and Shih C: Flora Reipublicae Sinicae. Tomus 74. Science Press, Beijing, p348, 1985.
17. Duke JA, Bogenschutz-Godwin MJ and Ottesen AR: Dukes Hand Book of Medicinal Plants of Latin America. CRC Press, Boca Raton, London, New York, p225, 2009.
18. Bohlmann F and Wegner P: Three diterpenes from *Conyza podoccephala*. *Phytochemistry* 21: 1693-1695, 1982.
19. Xu LP, Guo DA, Liu JS and Zheng JH: A New transclerodane diterpene lactone from *Conyza blinii*. *Heterocycles* 51: 3605-3610, 1999.
20. Mukhtar N, Iqbal K, Anis I and Malik A: Sphingolipids from *Conyza canadensis*. *Phytochemistry* 61: 1005-1008, 2002.
21. Mukhtar N, Iqbal K and Malik A: Novel sphingolipids from *Conyza canadensis*. *Chem Pharm Bull (Tokyo)* 50: 1558-1560, 2002.

22. Lenfeld J, Motl O and Trka A: Anti-inflammatory activity of extracts from *Conyza canadensis*. *Pharmazie* 41: 268-269, 1986.
23. Czeczot H, Tudek B, Kusztalak J, Szymczyk T, Dobrowolska B, Glinkowska G, Malinowski J and Strzelecka H: Isolation and studies of the mutagenic activity in the Ames test of flavonoids naturally occurring in medical herbs. *Mutat Res* 240: 209-216, 1990.
24. Ding Y, Su Y, Guo H, Yang F, Mao H, Gao X, Zhu Z and Tu G: Phenylpropanoyl esters from Horseweed (*Conyza canadensis*) and their inhibitory effects on catecholamine secretion. *J Nat Prod* 73: 270-274, 2010.
25. Hrutfiord BF, Hatheway WH and Smith DB: Essential oil of *Conyza canadensis*. *Phytochemistry* 27: 1858-1860, 1988.
26. Slater AF, Stefan C, Nobel I, van den Dobbelen DJ and Orrenius S: Signalling mechanisms and oxidative stress in apoptosis. *Toxicol Lett* 82-83: 149-153, 1995.
27. Qiao L and Wong BC: Targeting apoptosis as an approach for gastrointestinal cancer therapy. *Drug Resist Updat* 12: 55-64, 2009.
28. Huang HL, Fang LW, Lu SP, Chou CK, Luh TY and Lai MZ: DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation. *Oncogene* 22: 8168-8177, 2003.
29. Wu K, Nie Y, Guo C, Chen Y, Ding J and Fan D: Molecular basis of therapeutic approaches to gastric cancer. *J Gastroenterol Hepatol* 24: 37-41, 2009.
30. Mao Y, Song G, Cai Q, Liu M, Luo H, Shi M, Ouyang G and Bao S: Hydrogen peroxide-induced apoptosis in human gastric carcinoma MGC803 cells. *Cell Biol Int* 30: 332-337, 2006.
31. Martín R, Ibeas E, Carvalho-Tavares J, Hernández M, Ruiz-Gutierrez V and Nieto ML: Natural triterpenic diols promote apoptosis in astrocytoma cells through ROS-mediated mitochondrial depolarization and JNK activation. *PLoS One* 4: e5975, 2009.

RETRACTED