รายงานวิจัยฉบับสมบูรณ์

โครงการผลของสารต้านอนุมูลอิสระต่อการตายแบบอะพอพโทสิสของเซลล์มะเร็งเม็ดเลือดขาวที่ถูกกระตุ้นโดยขมิ้น

Antioxidant effect on HL-60 cell apoptosis induced by curcumin

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ธันวาคม 2547

สัญญาเลขที่ TRG4580091

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Abstract

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Project Title: Antioxidant effect on HL-60 cell apoptosis induced by curcumin

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Curcumin is the main biologically active phytochemical compound in turmeric. Curcumin has anti-inflammatory, antioxidant, anticarcinogenic and antiproliferative activities. The research aims were to identify the mode and mechanism of cell death of human promyelocytic leukemic (HL-60) cells induced by curcumin and to determine the effects of vitamin C, Trolox, reduced glutathione (GSH) and *N*-acetylcysteine (NAC) on this process. HL-60 cells were incubated with curcumin for 4 and 24 h and apoptotic cells were quantitated by flow cytometry following staining with annexin V-FITC and propidium iodide. Curcumin induced apoptosis in a dose-dependent manner over the concentration range 2.5-25 μM, which was also confirmed by the decrease in mitochondrial membrane potential. PI3K inhibitor (LY294002) and MAPKK/MEK inhibitor (PD98059) had different effect on the apoptotic cell death induced by curcumin (20 μM). There was an increase in free radical generation, as measured by dichlorofluorescein diacetate and flow cytometry, indicating the existence of oxidative stress in curcumin-treated HL-60 cells. In the presence of 10 μM curcumin, vitamin C (56, 560 nM and 5.6 μM) and GSH (1, 10 and 100 μM) reduced the number of apoptotic cells, but NAC and Trolox had a dual effect, being protective at 1, 10 μM, and 1 mM for NAC and 1 μM, for Trolox; and synergistic at 100 μM for NAC and 10, 100 μM, and 1 mM for Trolox.

Keywords: apoptosis, curcumin, HL-60 cells, antioxidants

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ชื่อโครงการ: ผลของสารต้านอนุมูลอิสระต่อการตายแบบอะพอพโทสิสของเซลล์มะเร็งเม็ดเลือดขาวที่ถูกกระตุ้นโดยขมิ้น

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เคอร์คิวมินเป็นสารสำคัญที่มีฤทธิ์ทางชีวภาพในเหง้าของขมิ้น เคอร์คิวมินเป็นสารต้านการอักเสบ ต้านอนุมูลอิสระ ต้านมะเร็ง และมีฤทธิ์ต้านการแบ่งตัวเพิ่มจำนวนของเซลล์ วัตถุประสงค์ของงานวิจัยนี้คือ การศึกษาชนิดของการตาย กลไกการตายของเซลล์มะเร็งเม็ดเลือดขาว (HL-60) ที่กระตุ้นโดยเคอร์คิวมิน และผลของไวตามินซี โทรล็อกซ์ กลูตาไธโอน (GSH) และเอ็นอะเซติลซิสเตอีน (NAC) ต่อการตายดังกล่าว เซลล์มะเร็งเม็ดเลือดขาวถูกบ่มด้วยเคอร์คิวมินเป็นเวลา 4 และ 24 ชั่วโมงและนำมาย้อมเซลล์ด้วยแอนเน็กซินไฟว์-เอฟไอทีซี และโพรพิเดียมไอโอไดด์และตรวจวัดการตายด้วยวิธีโฟลไซโตเมทรี เคอร์คิวมินกระตุ้นการตายแบบอะพอพโทสิสโดยขึ้นกับความเข้มข้นในช่วง 2.5-25 ไมโครโมลาร์ ซึ่งยืนยันว่าเป็นการตายแบบอะพอพโทสิสด้วยการลดลงของไมโตคอนเดรียลเมมเบรนโพเทนเชียล สาร PI3K (LY294002) และสารยับยั้ง MAPKK/MEK (PD98059) มีผลต่อการตายแบบอะพอพโทสิสที่กระตุ้นโดยเคอร์คิวมิน (20 ไมโครโมลาร์) มีการสร้างสารอนุมูลอิสระวัดโดยใช้ไดคลอโรฟลูโอเรสซีน ไดอะซีเตท และวิธีโฟลไซโตเมทรี พบว่าในเซลล์ HL-60 มีภาวะเครียดออกซิเดชันเกิดขึ้น ในภาวะที่ให้เคอร์คิวมิน (10 ไมโครโมลาร์) ไวตามินซี (56, 560 นาโนโมลาร์ และ 5.6 ไมโครโมลาร์) และ GSH (1, 10 และ 100 ไมโครโมลาร์) จะลดจำนวนเซลล์ที่ตายแบบอะพอพโทสิส แต่ NAC และโทรล็อกซ์มีผลสองแบบ คือ ลดจำนวนเซลล์ที่ตายแบบอะพอพโทสิสที่ความเข้มข้นของ NAC เท่ากับ 1, 10 ไมโครโมลาร์ และ 1 มิลลิโมลาร์ และที่ความเข้มข้นของโทรล็อกซ์ 1 ไมโครโมลาร์ และเสริมฤทธิ์เพิ่มการตายที่ความเข้มข้นของ NAC 100 ไมโครโมลาร์ และที่ 10, 100 ไมโครโมลาร์และ 1 มิลลิโมลาร์ของโทรล็อกซ์

คำสำคัญ: การตายแบบอะพอพโทสิส, เคอร์คิวมิน, เซลล์ HL-60, สารต้านอนุมูลอิสระ

Introduction

Apoptosis is a physiological suicide mechanism that preserves homeostasis, in which cell death naturally occurs during tissue turnover (1). It is the most common form of eukaryotic cell death. In general, cells undergoing apoptosis display profound structural changes, including a rapid blebbing of the plasma membrane and nuclear disintegration. The nuclear collapse is associated with extensive damage to chromatin and DNA cleavage into oligonucleosomal-length DNA fragments after activation of calcium-dependent endogenous endonucleases (2). Apoptosis is essential in many physiological processes, including the embryonic development and the maturation of immune system (3). It is currently the subject of intense research, partially because tumor cells are susceptible to death by apoptosis in response to drugs and/or radiation treatment. In the past few years the interest for using apoptosis as a possible measure of radiosensitivity and chemotherapeutic application has increased substantially both with regard to possibilities of using the extent of apoptosis as a biological dosimeter and for estimating the radiosensitivity and chemosensitivity of cancer before radiotherapy and chemotherapy (4).

Curcuma longa Linn. is a perennial herb originally cultivated widely in tropical regions of Asia from which dried rhizome is isolated the spice turmeric. It belongs to the family Zingiberaceae and has a long and distinguished human use in Eastern civilization. Its rhizome is used extensively. Turmeric, a powder from the dried rhizomes, is used for medicinal purposes and is reportedly used as an antiseptic, a cure for poisoning, to eliminate body waste products, for treating dyspepsia, and respiratory disorders, as a cure for some skin diseases, including wound healing, and as a house hold remedy for treating sprains and swellings caused by injury (5).

Curcumin, also known as diferuloylmethane (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), is the major yellow pigment extracted from turmeric, which is used extensively in curries. Its properties as a coloring and flavoring agent have led to uses as a dietary additive in variety of foods. These include saffron, mustard and other spices, gelatins, puddings, ice creams, soups, meats, pickles, margarine, and both alcoholic and nonalcoholic beverages (5, 6). Extracts containing curcumin have also been used in medicines in India and Southeast Asia for generations, and according to tradition are useful in the treatment of inflammation, skin wounds, hepatic and biliary disorders, cough, and coryza as well

as certain tumors (7). Curcumin has been identified to possess antimicrobial, anticancer, anti-inflammatory and topoisomerase-inhibitory activities, and has been reported to be an antioxidant and free-radical scavenger (5, 8-11). As a result, dietary intake of curcumin is especially high in these areas of Asia, where adults consume up to >200 mg of curcumin/day or up to 7-8 μmol/kg of body weight. Even in France, however, where curcumin exposure may be more representative of that typical worldwide, intake of as much as >3.4 μmol/kg/day has been documented (12).

The exposure of population worldwide to curcumin, and its many uses, has led to studies aimed at elucidating some of its activities. Curcumin and related compounds inhibit free radical generation and act as free radical scavengers and antioxidants, inhibit lipid peroxidation and oxidative DNA damage. Inhibition of lipoxygenase and cyclooxygenase resulting in decreased arachidonic acid release and metabolism, along with abilities to inhibit activation of NF-κB (13), may contribute to the anti-inflammatory activity of these compounds. Another property ascribed to curcumin is that of inhibition of c-jun/AP-1 (14) function and JNK activation (15). Curcuminoids have been noted to be potent inhibitors of cytochrome P450 and have the ability to induce glutathione S-transferase, and as such, have been proposed as potential chemoprotective agents (16). Because curcumin inhibits tumor formation in several murine tissues and antagonizes both initiation and promotion of tumors in rodent epithelial and colon cancer models (5, 6), interest has been raised in this compound as a chemopreventive agent (17). Recently curcumin has led to several Phase I human trials that have shown this agent to be tolerated well (18) and their successful completion suggests that curcumin use may increase in the future.

Curcumin's chemopreventive activity in animal model systems has led investigators to study its potential impact upon tumor cell growth and apoptosis. Several reports document an antiproliferative effect on cultured cells such as on colon cancer and breast cancer cells (19, 20). This may, in part, be because of programmed cell death since at high concentrations curcumin can induce apoptosis such as in human leukemia cells. In contrast, in other systems curcumin can inhibit apoptosis such as in T lymphocytes, and it protected rat lungs from injury by bleomycin and rat myocardium from adriamycin, respectively, but its impact on the therapeutic applications of antineoplastic drugs has not been well studied. Because reactive oxygen species (ROS) have been felt to play an important roles in drug-induced

apoptosis (21), one might suspect that curcumin, as an antioxidant, free radical scavenger, would inhibit the ability of chemotherapeutic drugs to induce apoptosis. Furthermore, curcumin inhibits JNK activation, which has been associated with chemotherapy-mediated induction of apoptosis in tumor cells (22).

It has been demonstrated that topical application of curcumin inhibits benzo(a) pyrene-induced DNA adduct formation, and development of skin tumors as well as TPA-induced epidermal DNA synthesis and tumor promotion in mouse skin (23). In addition, a tumoricidal activity of curcumin has been observed in a wide range of cell lines like NIH3T3, mouse sarcoma S180, human kidney cancer cell 293 (24), Chinese hamster ovary (8) and human basal cell carcinoma (25). Furthermore, curcuma has a strong inhibitory effect on cell proliferation in the HT-29 and HCT-15 human colon cancer cell lines (26). Importantly, dietary administration of curcumin during initiation and/or postinitiation periods significantly suppresses development of chemically induced forestomach, duodenal, and colon tumors in CF-1 mice (27); it also reduces formation of focal areas of dysplasia and aberrant crypt foci in the colon that are early preneoplastic lesions in rodents (28, 29). Curcumin also has potential as an antiviral agent. Indeed, it has been proven to be a modest inhibitor of HIV-1 and HIV-2 proteases. Curcumin has also been shown to induce apoptosis in numerous animal and human cells, although the cell death pathway depends very much on cell type (24, 25, 30-34).

It has been found that curcumin can reduce the number of cells with chromosomal aberrations but protects normal cells from chromatid breaks due to exposure to gamma-irradiation (35). However, the mechanisms underlying these diverse effects of curcumin are not fully understood. Among the possibilities, regulation of an array of cellular biochemical processes by curcumin such as inhibition of nitric oxide synthase, receptor tyrosine kinase and protein kinase C (36-38) activities and the alteration of transcriptional factors c-jun/AP-1, nuclear factor kappaB, and p53 by curcumin have been suggested (13, 24). Recently it has also been suggested that production of reactive oxygen intermediates may be the cause of tumor cell apoptosis as a result of curcumin treatment (30). Curcumin also has been reported to induce mitochondrial abnormalities promote p53-dependent apoptosis and activation of caspase-8 and caspase-3 (25, 39-41).

Vitamin C also known as, L-ascorbic acid and dehydroascorbic acid, is a much talked about vitamin, with people claiming it as a cure-all for many diseases and

problems, from cancer to the common cold. Yet, this miracle vitamin cannot be manufactured by the body, and needs to be ingested. Vitamin C is required in the synthesis of collagen in connective tissue, neurotransmitters, steroid hormones, carnitine, conversion of cholesterol to bile acids and enhances iron bioavailability. Ascorbic acid is a great antioxidant and helps protect the body against pollutants. Because vitamin C is a biological reducing agent, it is also linked to prevention of degenerative diseases, such as cataracts, certain cancers and cardiovascular diseases. Ascorbic acid also promotes healthy cell development, proper calcium absorption, normal tissue growth and repair - such as healing of wounds and burns. It assists in the prevention of blood clotting and bruising, and strengthening the walls of the capillaries (42).

Trolox is a cell–permeable, water-soluble derivative of vitamin E with potent antioxidant properties. It can prevent peroxynitrite–mediated oxidative stress and apoptosis in rat thymocytes. Alpha-tocopherol is the most active form of vitamin E in humans, and is a powerful biological antioxidant. Antioxidants such as vitamin E act to protect your cells against the effects of free radicals, which are potentially damaging by-products of the body's metabolism. Free radicals can cause cell damage that may contribute to the development of cardiovascular disease and cancer. Studies are underway to determine whether vitamin E might help prevent or delay the development of those chronic diseases (42).

Glutathione is the major endogenous antioxidant produced by the cell. It is a tripeptide, which composes of glutamate, cysteine and glycine. Glutathione participates directly in the neutralization of free radicals, reactive oxygen compounds, and maintains exogenous antioxidants such as vitamins C and E in their reduced (active) forms. In addition, through direct conjugation, glutathione plays a role in the detoxification of many xenobiotics (foreign compounds) both organic and inorganic. Glutathione is an essential component of the human immune response. Proposed mechanisms of immune enhancement include: (1) optimizing macrophage functions, (2) offsetting oxidative damage associated with lymphocyte monoclonal expansion, and (3) stabilizing the mitochondrial membrane thereby, reducing apoptosis in lymphocytes (42).

N-acetyl-L-cysteine (NAC) is an amine protected version of cysteine that is rapidly hydrolyzed in the body to the amino acid cysteine. NAC supplements are

moderately effective, but dosing is limited due to toxic side effects (such as headache, dizziness, blurred vision) associated with cysteine supplementation (42).

The aims of this study were to investigate the roles of antioxidants both single and combined effect on HL-60 cell apoptosis induced by curcumin, and to study the mechanism of signal transduction in such system. We also investigated the effect of curcuminoid derivatives found in the curcumin on HL-60 cells, i.e., demethoxycurcumin and bisdemethoxycurcumin. We measured the apoptotic cells by using annexin V-FITC and propidium iodide staining and processed through flow cytometer. The mitochondrial membrane potential was also measured in the system, which was induced by curcumin. Phosphotidylinositol 3-kinase (PI3K) inhibitor and MAP kinase kinase inhibitor were applied to demonstrate their effect on curcumin-induced apoptosis in HL-60 cells.

Materials and Methods

RPMI-1640 medium and fetal bovine serum were obtained from Gibco-BRL, New York, NY, USA. Annexin V-FITC kit was obtained from Roche, Indianapolis, IN, USA. Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), glutathione, N-acetylcysteine, vitamin C, 2',7'-dichlorofluorescein diacetate (DCFH-DA), and 3,3'-dihexyloxacarbocyanine iodide or $DiO_6(3)$ were obtained from Sigma, St. Louis, MD, USA. Trolox was obtained from Aldrich, Milw, WI, USA. LY294002 and PD98059 were obtained from Calbiochem, La Jolla, CA, USA.

Curcuminoids (alcoholic and isopropanol extract), demethoxycurcumin, bisdemethoxycurcumin and curcumin I were generous gifts from Dr. Porn-ngarm Limtrakul.

Cell culture and treatment conditions

HL60 cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 µg/ml). The preconfluent (growth phase) cells were treated with curcumin at the various concentrations for 4, 24 or 48 hours. Diluted curcumin solutions were prepared in alcohol (0.5% final concentration). It was found that 0.5% alcohol did not affect cell viability. After incubation the cells with curcumin, the cells were processed through agarose gel electrophoresis and flow cytometry as indicated.

Treatment conditions

Preconfluent HL60 cells ($1x10^6$ cells) were treated with hydrogen peroxide at the concentrations of 0.1, 1, 10 mM, for 30 min at 37 °C under an atmosphere of 5% CO_2. Control cells were incubated in the absence of hydrogen peroxide. At the termination of the incubation, catalase was added (final concentration of 100 units/ml). Cell morphology was examined under phase contrast microscope and fluorescence microscope after staining with propidium iodide. HL60 cells were also collected for the agarose gel electrophoresis and flow cytometry

In the conditions of treatment with antioxidant(s), i.e., vitamin C or Trolox or N-acetylcysteine or glutathione, was (were) added simultaneously with curcumin.

Vitamin C was varied at the concentration of 56, 560 nM, 5.6, and 56 μM. For Trolox, the concentration was varied from 1, 10, 100 μM, to 1 mM. For N-acetylcysteine, the concentrations was 1, 10, 100 μM, and 1 mM. For glutathione, the dose was 1, 10, 100 μM, and 1 mM.

Then two kinds of antioxidants were added to the cell culture system to see the combined effect on HL60 cell apoptosis induced by curcumin (10 μM). Then the cells were processed for agarose gel electrophoresis and flow cytometry.

HL-60 cells were pretreated with LY294002 or PD98059 at 10, 20 and 50 μM for 50 minutes and then with curcumin at 20 μM for 4 hours or with curcumin (20 μ M) alone or with LY294002 (50 μM) alone or PD98059 (50 μM) alone for 4 hours. Then $DiOC_6(3)$ were added at 40 nM for 15 minutes at 37 °C and was measured for mitochondrial membrane potential.

Agarose gel electrophoresis (43)

After HL60 cells were treated with H_2O_2, or curcumin and/or other antioxidants, the cells were collected and centrifuged down at 200 x to yield the cell pellets. The precipitated cells were used for further process. The cells were lysed by using 0.25% Igepal in TBE (Tris-borate EDTA buffer). Then ribonuclease A (RNase A was added to the final concentration of 10 μg/ml, incubated at 37 °C for 30 min. After that, the proteinase K was put into the lysate to the concentration of 100 μg/ml, and incubated at 37 °C for further 30 min. The loading dye buffer was finally added to the mixture of cell lysate and was then applied to 2 % agarose gel. The electrophoresis was processed in the condition of 60 volts for 4 h, then the gel was stained with ethidium bromide for 15 min and destained with distilled water for 30 min. Finally the DNA cleavage band or ladder pattern was observed under UV transilluminator.

If the bands were so smeared due to the ribonucleic acid (RNA), the smear could be got rid off by incubating with the RNase (20 mg/ml) in TE (Tris-EDTA) buffer overnight.

Flow cytometry (44)

HL60 cells were collected at the concentration of 10^6 cells. The cells were washed once and then centrifuged at 200 x g to get the cell pellets which were

resuspended in 100 µl of the binding buffer provided by the reagent kit. Annexin V-FITC (2 µl) and propidium (2 µl) were added in each tube. Then they were incubated at room temperature for 15 min in the dark. Finally the binding buffer, 900 µl, were put into it and mixed for further processing.

Mitochondrial membrane potential and reactive oxygen species (ROS) measurement (45)

For mitochondrial membrane potential ($\Delta\Psi$) and intracellular ROS measurement, $5x10^5$ cells were incubated for 15 minutes at 37 °C with 3,3'-dihexyloxacarbocyanine iodide [$DiO_6(3)$, 40 nM], or 2',7'-dichlorofluorescein diacetate (DCFH-DA, 5 µM) followed by FACScan (Becton Dickinson) analysis.

Statistics

The duplicate tests were performed in three independent experiments and analyzed based on Kruskal Wallis analysis (one way ANOVA). For the two variables the data were analyzed by using two way ANOVA.

Results

The effect of curcuminoids and curcumin on HL-60 cell apoptosis

When HL-60 cells were treated with curcumin (diferuloylmethane) for 4 hours at various concentrations, it was found that the cells died via apoptosis in a dose dependent manner as shown in Fig. 1. The apoptotic cells were the cells that were stained with annexin V-FITC. The percentage of apoptotic cells was increased according to the concentrations of curcumin used. In control (without treatment) the cells died 12.39%, when treated with curcumin at 10, 20, and 30 μM, the percentages of apoptotic cells were 17.54, 22.77, and 25.22%, respectively.

The dot plots of the effect of curcuminoid (isopropanol extract) on HL-60 cell apoptosis at various concentrations were shown in Fig. 2. The data was analyzed, averaged and finally presented as bar graph in Fig. 5. The apoptotic cell death increased in a dose dependent manner with the highest response at 10 μg/ml or 25 μ M. At 25 nM (10 ng/ml), 250 nM (100 ng/ml), 2.5 μM (1 μg/ml) and 25 μM (10 μ g/ml) gave almost the same percentage of apoptotic cells, i.e., almost 30%) as shown in Fig. 5A.

When HL-60 cells were treated with curcumin for 24 hours at different concentrations, the cells underwent apoptosis as shown in Fig. 3. At concentration of curcumin (10 μg/ml or 25 μM), the cells died via apoptosis almost 50% whereas at 0, 1, 10, 100 ng/ml and 1 μg/ml the cells died about 20%. When analyzed the data with Kruskal Wallis analysis (one way ANOVA), it was statistically not different ($p=0.061$).

When the cells were incubated with curcumin for 24 and 48 hours, it was found that the percentages of apoptotic cells were not different at concentrations of curcumin at 0, 1, 10, 100 ng/ml and 1 μg/ml. But the cells died more at 10 μg/ml when incubated for 48 hours when compared to 24 hours, which were 70% for 2 days of incubation and almost 50% for 1 day as shown in Fig. 4. When the data were analyzed by using two way ANOVA, it was significantly different ($p<0.05$).

When the cells were incubated with curcuminoid (isopropanol extract) for a day, the result was shown in Fig. 5A as mentioned in the dot plot diagram of Fig. 2. When the data in Fig. 5A were analyzed by using one way ANOVA, it was not significantly different ($p=0.179$). The data of Fig. 5B which were the comparison of

the number of apoptotic cells incubated for 24 and 48 h, were analyzed by two way ANOVA and it was statistically not different (p=0.110).

The percentage of apoptotic cells when induced with curcuminoid (alcoholic extract) was shown in Fig. 6A. Of note the percentage of cell death was increased from 1, 10, 100 ng/ml to 1 μg/ml but decreased at 10 μg/ml which was not significant differently when analyzed by one way ANOVA (p=0.345). When compared the number of apoptotic cells between day 1 and day 2 (Fig. 6B), it was also not significantly different (two way ANOVA) (p=0.757).

As shown in Fig. 3 that the number of apoptotic cells was markedly increased between 1 μg/ml (2.5 μM) to10 μg/ml (25 μM), so we varied the concentrations in the range of these values. When HL-60 cells were incubated with curcumin, the cells underwent apoptosis in a dose dependent manner (from 2.5, 5, 10, 20, and 25 μM) as shown in Fig. 7. It was statistically different (p=0.02) from Kruskal Wallis analysis or one way ANOVA. Of note, at lower concentrations (2.5 μM, 5 μM, 10 μM) curcumin inhibited apoptotic cells when compared to control (Fig. 7).

The effect of antioxidants on HL-60 cell apoptosis

When the HL-60 cells were treated with vitamin C, compared to those without treatment, it was found that vitamin C did not cause the cells to die much more than control. But when the cells were treated with curcumin simultaneously, it decreased the number of cell death (apoptosis) at doses of 56 nM, 560 nM and 5.6 μM compared to treatment with curcumin (10 μM) alone. Thus, vitamin C had inhibitory effect on apoptotic cell death as shown in Fig. 8. It was statistically nonsignificant (p=0.261; Kruskal Wallis analysis).

When the cells were treated with vitamin C for 1 and 2 days, it was demonstrated that the percentages of apoptotic cells were increased at all concentrations of vitamin C (56, 560 nM, 5.6 and 56 μM) on day 2. This meant that vitamin C was not able to inhibit the apoptosis in these conditions (Fig. 9). This condition might cause the cell to die via apoptosis by itself and could not be prevented by vitamin C on day 2. The number of apoptotic cells on day 1 and day 2 was not significantly different at various concentrations of vitamin C (p=0.092 by two way ANOVA).

But when the HL-60 cells were treated with vitamin C first for 1 day and then with curcumin for another day, it was found that the percentage of apoptotic cells was increased when compared to without any treatment except at 56 nM (Fig. 10). The treatment of vitamin C first for a day could not prevent cells from apoptosis but in contrast, it increased the number of apoptotic cells at 560 nM, 5.6 and 56 μM (Fig. 10; gray bars). The pretreatment of vitamin C had a synergistic effect on apoptosis when induced with curcumin except at the concentration of vitamin C at 56 and 560 nM, which could reduce the number of cell death compared to treatment with curcumin alone (Fig. 10; black bars). Hence, at the concentrations of 56 and 560 nM of vitamin C, it could inhibit apoptotic cell death. However, when analyzed with two way ANOVA it was not significantly different ($p=0.126$).

Since curcumin is the compound composed of three forms of curcumin derivatives which are curcumin I, demethoxycurcumin and bisdemethoxycurcumin. When the HL-60 cells were treated with each derivative of curcumin (10 μM or 4 μg/ml), the isopropanol extract, and alcohol extract, it was found that the most potent form of curcuminoid, i.e., most cytotoxic to HL-60 cells, was demethoxycurcumin and the least toxic one was curcumin I as shown in Fig. 11. But when analyzed by using Kruskal Wallis analysis, it was not significantly different ($p=0.08$).

Another antioxidant compound that was used to test in the experiment was Trolox, which is a water-soluble vitamin E. It was found that when the HL-60 cells were treated with Trolox alone, it did not cause cells to die more at doses of 1, 10, and 100 μM but at concentration of 1 mM it caused the cells to undergo apoptosis 4 folds compared to control (without any treatment). However, in the status of treatment with curcumin 10 μM, it was found that at 10 μM, 100 μM, and 1 mM of Trolox, it caused the cells to undergo apoptosis more than the condition of treatment with curcumin 10 μM alone; whereas at the concentration at 1 μM it reduced the number of apoptotic cells. This meant that Trolox had dual effect. It was a prooxidant at 10 and 100 μM and 1 mM and showed the inhibitory effect of HL-60 cell apoptosis at 1 μM when compared to the number of apoptotic cells treated with curcumin alone as shown in Fig. 12. However, when analyzed by using Kruskal Wallis analysis, it was not significantly different ($p=0.060$).

Glutathione (GSH), an antioxidant found mainly in the red blood cells and also in other mammal cells, was used to test the inhibitory or stimulatory effect on the

system of HL-60 cell apoptosis induced by curcumin (10 μM). It was found that GSH could reduce the number of apoptotic cells compared to the system when treated with curcumin alone as shown in Fig. 13. It was statistically significant (p=0.031) by Kruskal Wallis analysis. When considering the effect of GSH alone on HL-60 cell apoptosis, it could reduced the number of apoptotic cells at 1, 10, and 100 μM compared to without any treatment..

The effect of N-acetylcysteine on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in Fig. 14. NAC had dual effect, i.e., at concentrations of 1, 10 μM and 1 mM, it reduced the number of cell death compared to that treated with curcumin alone. Meanwhile at concentration of 100 μM, NAC enhanced the number of apoptotic cells, i.e., it had synergistic effect with curcumin in inducing apoptosis in HL-60 cells. However, it was not significantly different (p=0.263) when analyzed by Kruskal Wallis analysis. When considering the effect of NAC alone (without curcumin treatment), it could decrease the number of apoptotic cells at 1, 10, 100 μM and 1 mM compared to without any treatment.

The combined effect of antioxidants on HL-60 cell apoptosis

The combined effect of vitamin C and Trolox on HL-60 apoptosis induced by curcumin (10 μM) was shown in Fig. 15. It was found that at concentration of 1 mM of Trolox, it enhanced the number of apoptotic cells significantly compared to that at concentrations of Trolox at 1, 10, and 100 μM. When analyzed by using two way ANOVA, it was shown that the number of apoptotic cells was significantly different in various concentrations of Trolox and at different concentrations of vitamin C (p=0.001).

The effect of Trolox and NAC on HL-60 cell apoptosis induced by curcumin (10 μM) was demonstrated in Fig. 16. At the concentration of Trolox at 1 mM it showed the synergistic effect to cause HL-60 cells to die more than the other concentrations of Trolox when combined with NAC. That meant at 1 mM of Trolox, it acted as a potent prooxidant. However, it was not statistically significant when analyzed by two way ANOVA (p=0.932).

The effect of Trolox and GSH on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in Fig. 17. The pattern of changes in apoptotic cells was similar to the previous ones, i.e. at 1 mM of Trolox, it enhanced the number of apoptotic cells

significantly and could be concluded that at this concentration, it is a prooxidant. It was significantly different when analyzed by two way ANOVA (p=0.001).

For the effect of GSH and NAC on HL-60 cell apoptosis induced by curcumin (10 μM), it was found that at 10 μM of GSH, it had the highest effect in enhancing the percentage of apoptotic cells, the second potent prooxidant was at 100 μM of GSH and the least potent concentration was at 1 μM. It was not in a dose dependent manner (when GSH concentration was considered) as shown in Fig. 18. When analyzed by two way ANOVA, it was not significantly different (p=0.322).

The effect of vitamin C and GSH on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in Fig. 19. It was found that at the concentration of vitamin C at 56 μM, it could increase the percentage of apoptotic cells whereas the second potent concentration of vitamin C was at 5.6 μM. However, it was not statistically different when analyzed by two way ANOVA (p=0.201).

The combined effect of vitamin C and NAC on HL-60 cell apoptosis induced by curcumin 10 mM, was shown in Fig. 20. At doses of NAC 1 and 10 μM and at concentration of vitamin C at 5.6 and 56 μM, HL-60 cells underwent apoptosis more than other concentrations but at vitamin C of 560 nM and NAC at 100 μM, the percentage of apoptotic cells was high as well. So, the number of apoptotic cells depended on the concentration of both vitamin C and NAC. The relationship of different concentrations of both vitamin C and NAC on HL-60 cell apoptosis was significantly different (p=0.002) when analyzed by two way ANOVA.

The mechanism of HL-60 cell apoptosis induced by curcumin

A reduction of mitochondrial membrane potential was noted within 4 hours of curcumin induction as shown in Fig. 21. Upper left picture showed the membrane potential of mitochondria in the HL-60 cells without curcumin treatment. When curcumin was added to the system at 10, 20, and 30 μM, there was a decrease in membrane potential in a dose response manner. Furthermore, when the incubation time with curcumin was increased to 24 hours, it was found that the decrease in membrane potential was more than at 4 hours which was in a time dependent manner as shown in Fig. 22.

Production of reactive oxygen species (ROS) including hydroxyl radicals and hydrogen peroxide measured by 2',7'-dichlorofluorescein diacetate or DCFH-DA was

affected by curcumin treatment as shown in Fig. 23. The left middle picture was the positive control, i.e. hydrogen peroxide (10 mM) treatment for 30 min and (open peak) overlay with the negative control in the left lower picture. However, at curcumin treatment at 20 and 30 μM the ROS production was decreased. This might be due to the secondary necrosis of the HL-60 cells that caused the ROS to be dispersed to the environment and could not be measured within the cells anymore. Alternatively, it might be because of the formation of fragmented bodies that caused the reduction of fluorescence of DCF.

When HL-60 cells were treated with 50 μM of LY294002, a PI3K inhibitor, for 50 minutes, then with curcumin (20 μM) for 4 hours, it enhanced the reduction in mitochondrial membrane potential to 46.29%. It meant that PI3K inhibitor increased the HL-60 cell apoptosis compared to that treated with curcumin alone (31.15%) as shown in Fig. 24.

Finally, when HL-60 cells were treated with PD98059 (a MEK or MAP kinase kinase inhibitor), it caused more reduction of mitochondrial membrane potential when compared to the system treated with curcumin (20 μM) alone as shown in Fig. 25. When treating the cells with PD98059 (10 μM) the mitochondrial membrane potential reduction was 40.02%, at 20 μM of PD98059 its reduction was 32.90%, and at PD98059 50 μM it was 32.89%. It seemed that at lower concentration of PD98059, it could cause or enhance the reduction of mitochondrial membrane potential more than higher dose. The most effectiveness of enhancement was at 10 μM of PD.

Agarose gel electropnoresis

There was no ladder pattern when the cells were treated with curcumin for 24 h at the concentrations of 1, 10, 100 ng/ml, 1, 2, 4, 8 and 10 μg/ml as shown in Fig. 26. This was confirmed as shown in Fig. 27 when treated the cells with curcumin at 1, 2, 4, and 8 μg/ml for 24 and 48 h. However, on repeating, it was found that the HL-60 cells which were treated with curcumin at 1 μg/ml for 48 h, produced ladder pattern as shown in Fig. 28 (lane 8).

It was found that when the HL-60 cells were treated with curcuminoid extract (isopropanol) at concentrations of 1, 10, 100 ng/ml, 1, and 10 μg/ml for 1 and 2 days, there was no ladder pattern detected (except positive control) as shown in Fig. 29. When the cells were treated with curcuminoid (alcoholic extract) at concentrations of

1, 10, 100 ng/ml, 1, and 10 μg/ml for 24 and 48 hours, there was no ladder pattern detected except lane 1 (positive control) as shown in Fig. 30.

The cells when treated with hydrogen peroxide in PBS compared to the cells treated with hydrogen peroxide in media at 0, 0.1, 1, and 10 mM; it was found that there were ladder pattern at hydrogen peroxide in media at concentrations of 0.1 and 1 mM as shown in Fig. 31 (lane 7 and 8). This was also confirmed in Fig 32 (lane 11).

The effect of vitamin C and curcumin in simultaneous treatment was shown in Fig. 32 (lane 3-5). The concentrations of vitamin C were 56, 560 nM and 5.6 μM in combination of treatment with curcumin (10 μM). The result was that there was no ladder pattern. In lane 6-8, the cells were treated with curcumin at 10, 15, and 30 μM, there was a band at the lowest molecular weight of the ladder pattern. This meant that there was an activity of DNase to cleave the DNA. This character might be an indication of fragmented DNA, however, it was not ladder-pattern.

In the conditions of treatment with vitamin C for 24 h and then followed with curcumin for another 24 h, it was found that at various concentrations of vitamin C (0, 56, 560 nM, 5.6 and 56 μM and in the status with curcumin 10 μM (as shown in Fig. 33 lane 10-13), there was a smear pattern of DNA on agarose gel electrophoresis compared to the simultaneous treatment in Fig. 33 (lane 3-6) which showed no ladder pattern but also did not contain smear pattern. From, the gel pattern it could be concluded that the cells died via necrosis or secondary necrosis when treatment with vitamin C for 24 h and then with curcumin (10 μM) for another 24 h as there was smear pattern (a characteristic of necrosis on DNA agarose gel electrophoresis).

The effect of GSH on HL-60 cell DNA pattern in the status with or without curcumin was shown in Fig. 34. There was no ladder pattern observed. The effect of Trolox in the presence or absence of curcumin on DNA pattern of HL-60 cells was shown in Fig. 35, there was no ladder pattern as well. Whereas the DNA pattern of the HL-60 cells when treated with vitamin C in the status with or without curcumin was demonstrated in Fig. 36, there was also no ladder pattern shown. Furthermore, the DNA agarose gel electrophoresis of HL-60 cells when treated with NAC in the presence or absence of curcumin was illustrated on Fig. 37, which no ladder pattern was shown as well.

Results

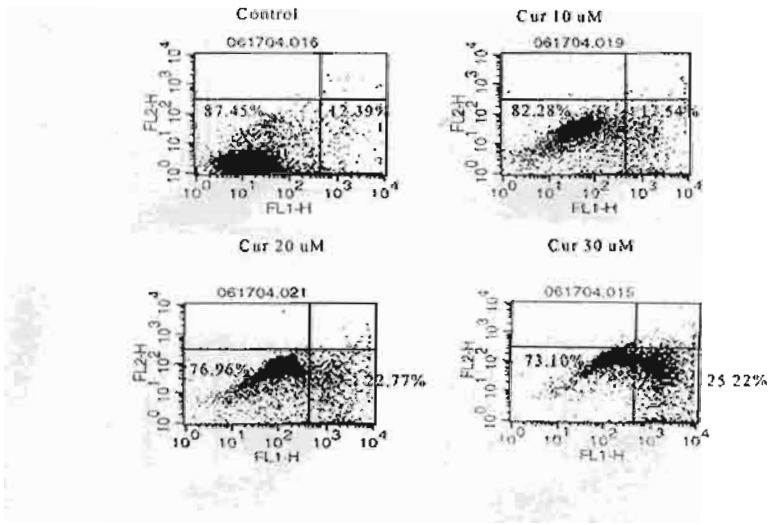


Fig. 1 The effect of curcumin at various concentrations (10, 20, and 30 μM) on HL-60 cell apoptosis incubated for 4 h. This is the representative of three experiments which were performed.

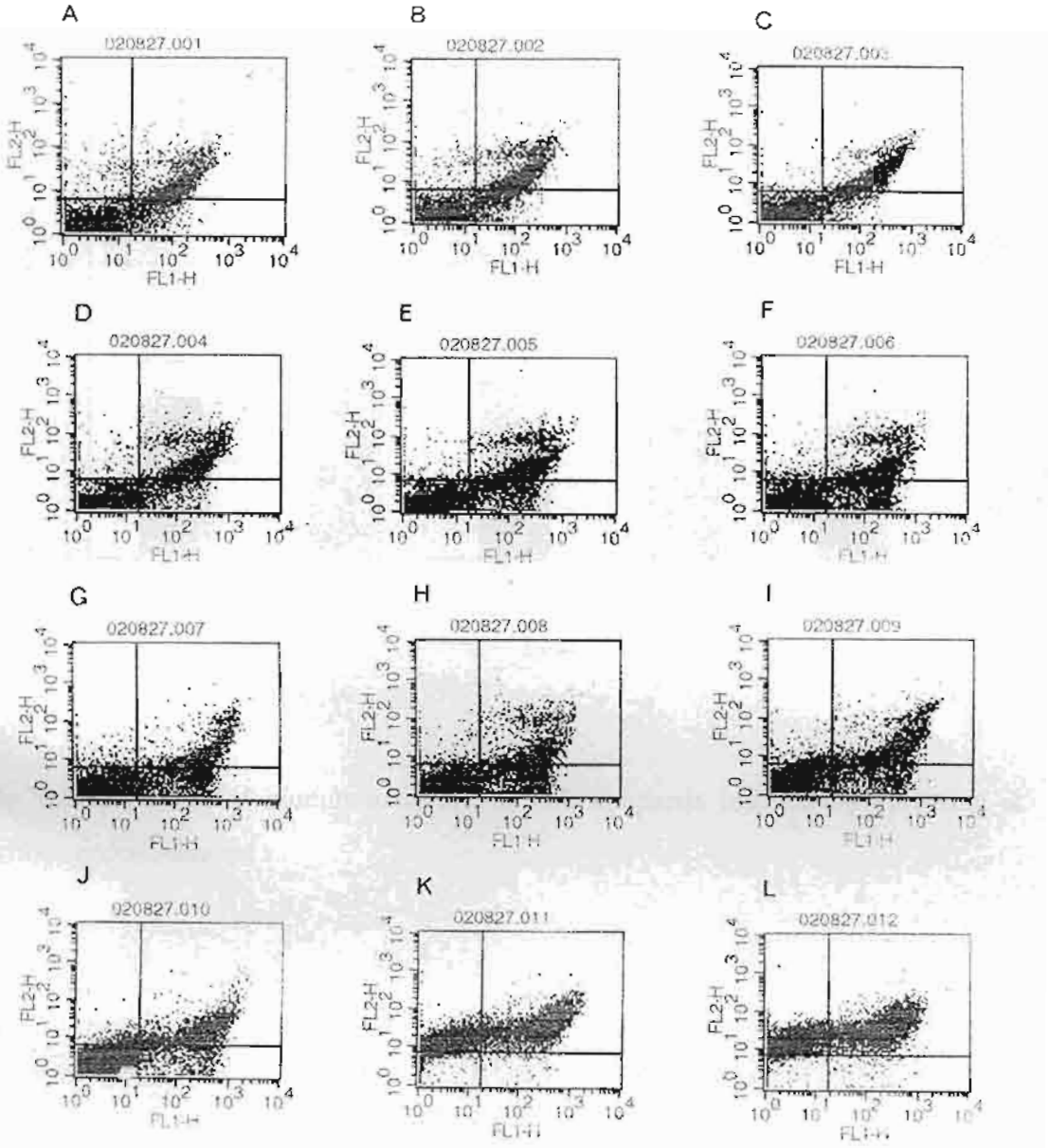


Fig. 2 Dot plots of the effect of curcuminoid (isopropanol extract) on HL-60 cell apoptosis at various concentrations for 24 h. A, B control or without treatment; C, D cells treated with curcuminoid (1 ng/ml); E, F cells treated with curcuminoid (10 ng/ml); G, H cells treated with curcuminoid (100 ng/ml); I, J cells treated with 1 μg/ml; K, L cells treated with 10 μg/ml of curcuminoid.

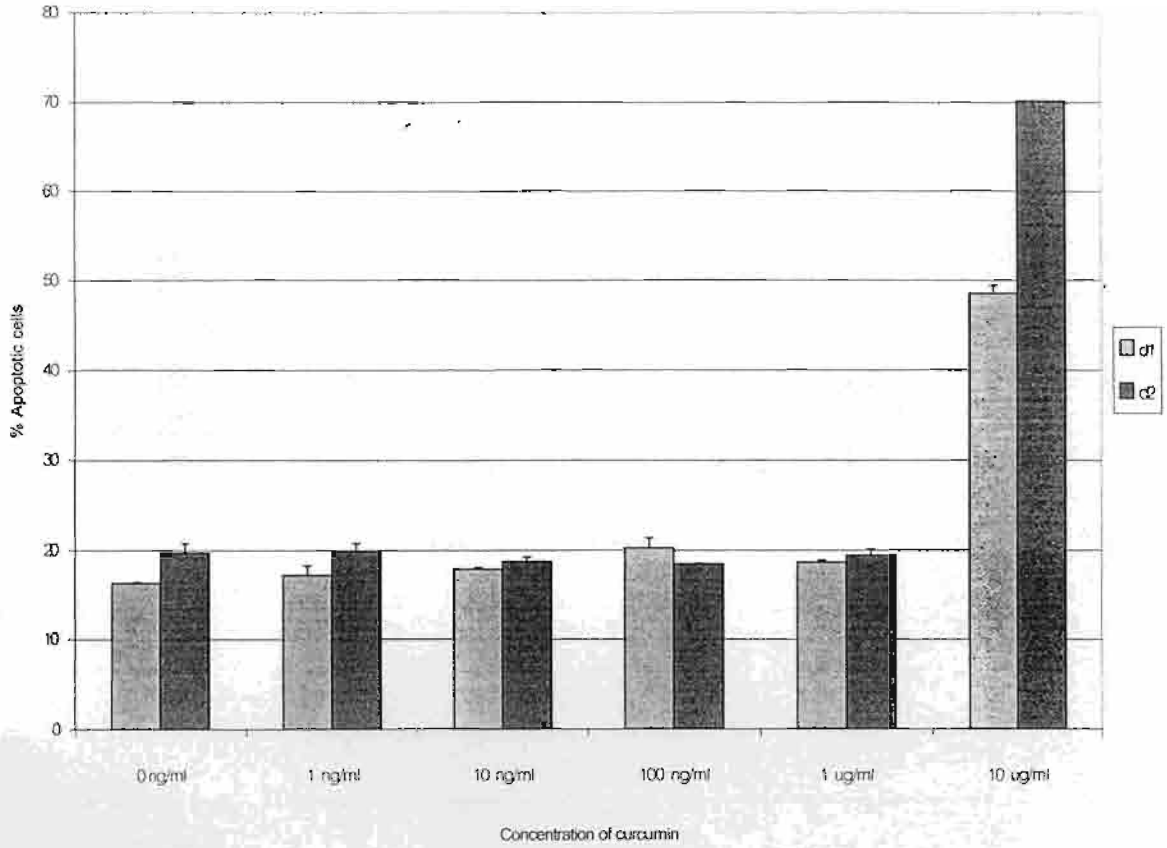
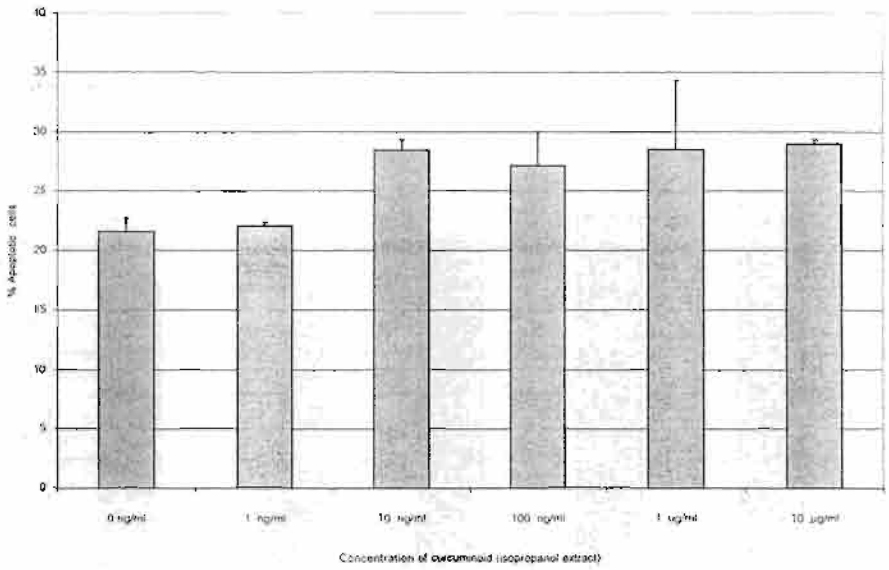


Fig. 4 The effect of curcumin on HL-60 cell apoptosis at various concentrations and at incubation time of 1 and 2 days.

A.



B.

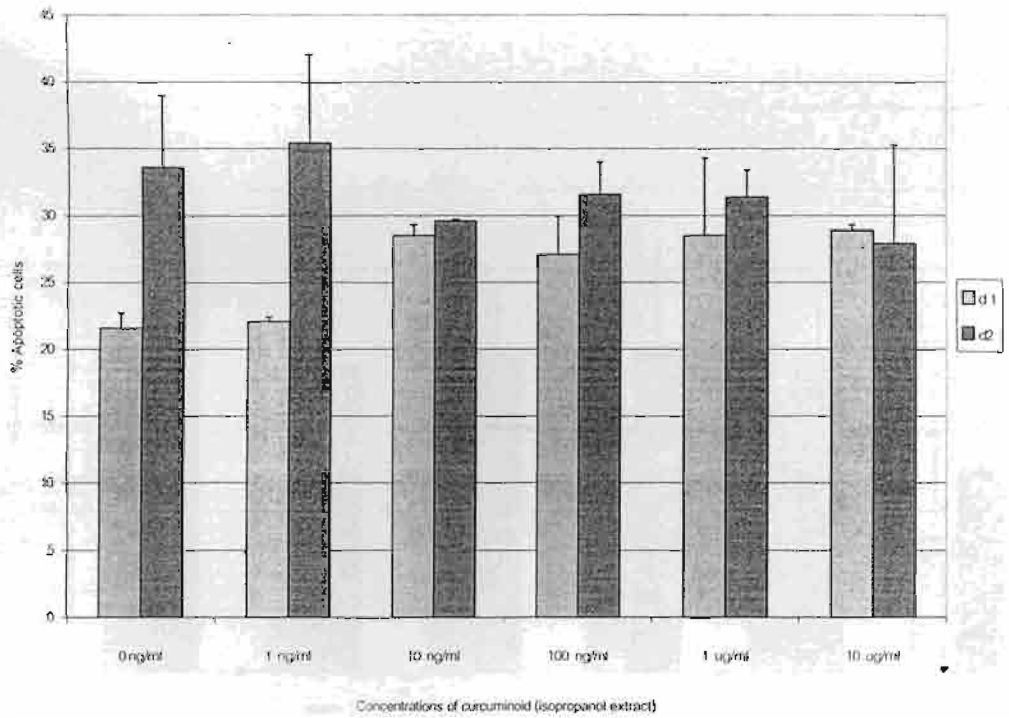
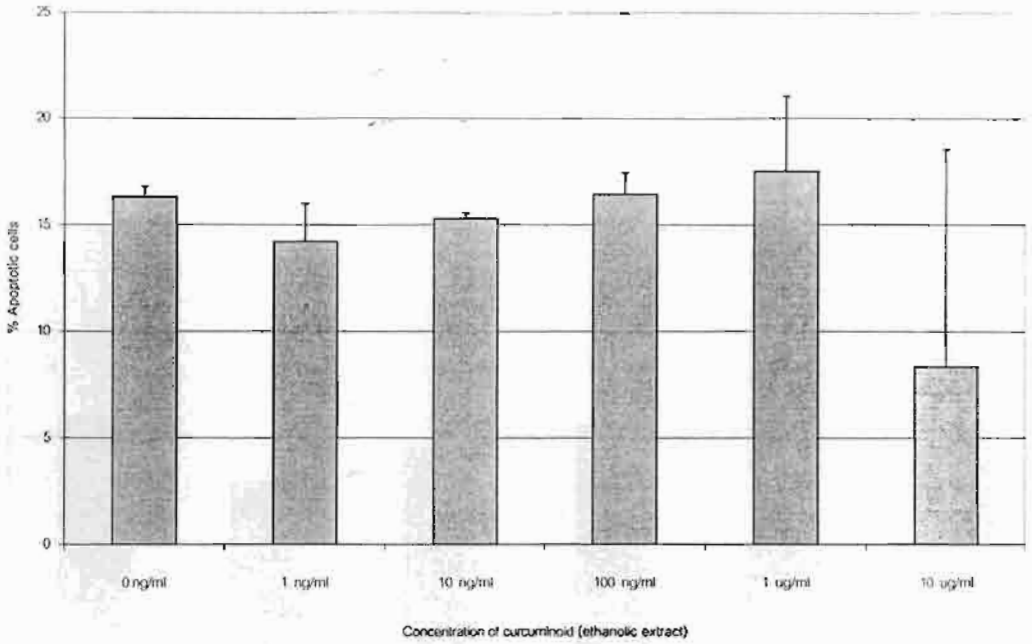


Fig. 5 The effect of curcuminoid extracts (isopropanol extract) on HL-60 cell apoptosis at various concentrations on day 1 (A) and comparison between day 1 and day 2 was shown in (B).

A.



B

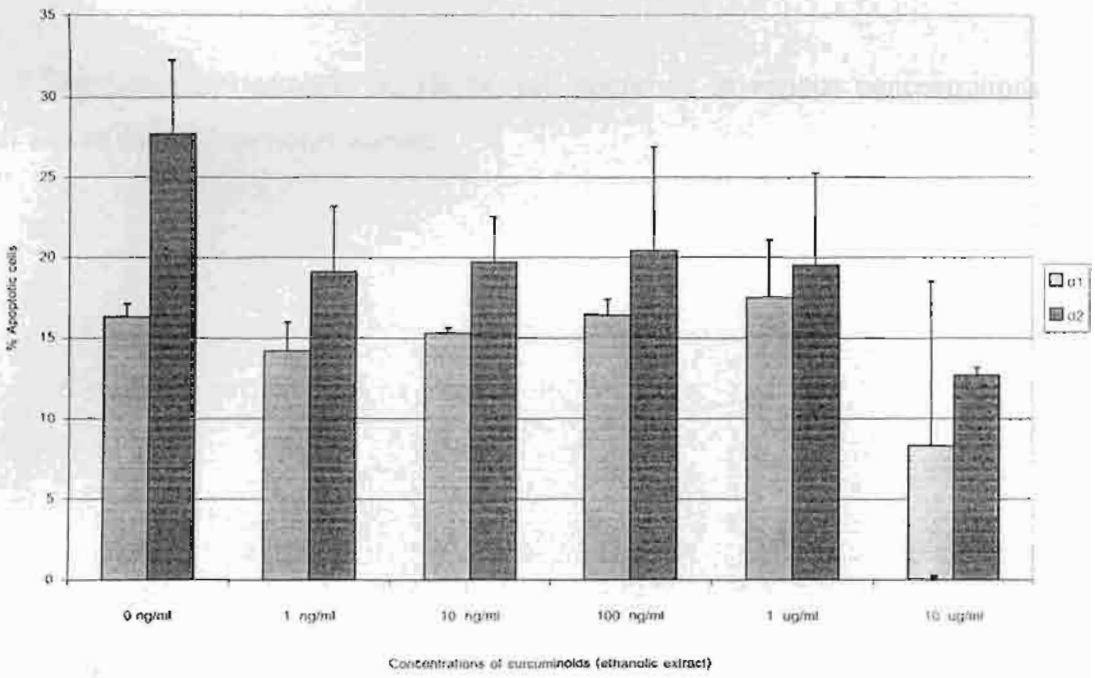


Fig. 6 The effect of curcuminoid extract (ethanolic extract) on HL-60 cell apoptosis at various concentrations on day 1 (A) and comparison between day 1 and day 2 was shown in (B).

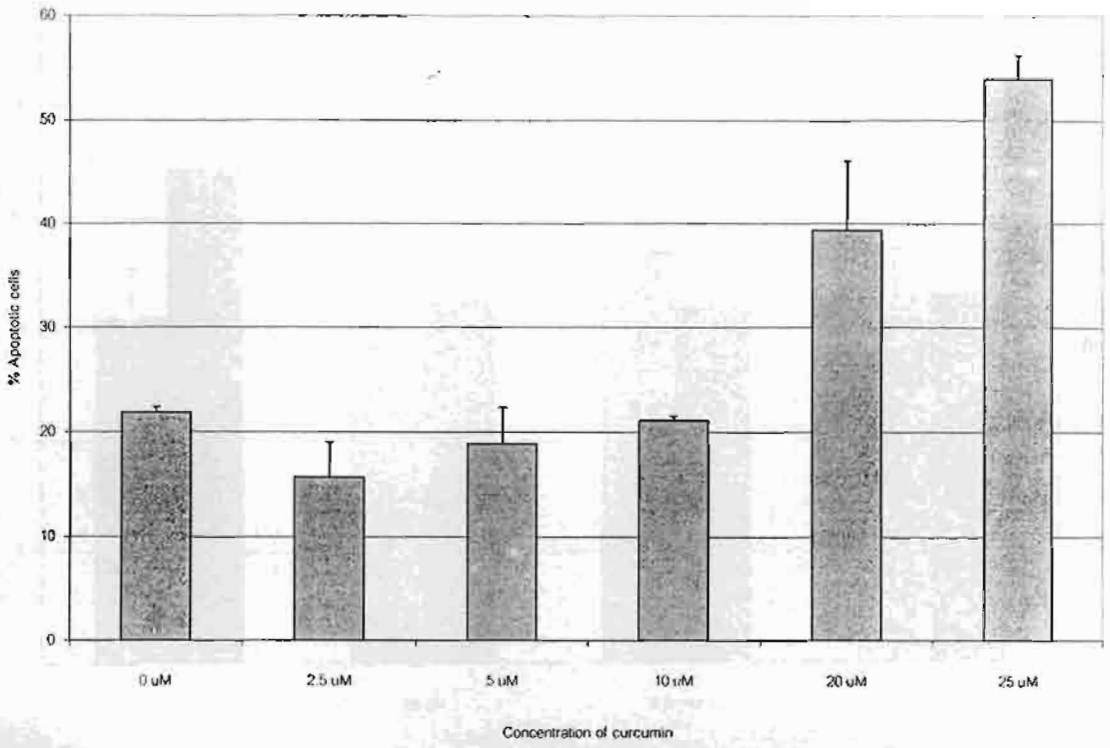


Fig. 7 The effect of curcumin on HL-60 cell apoptosis at various concentrations which was in a dose dependent manner.

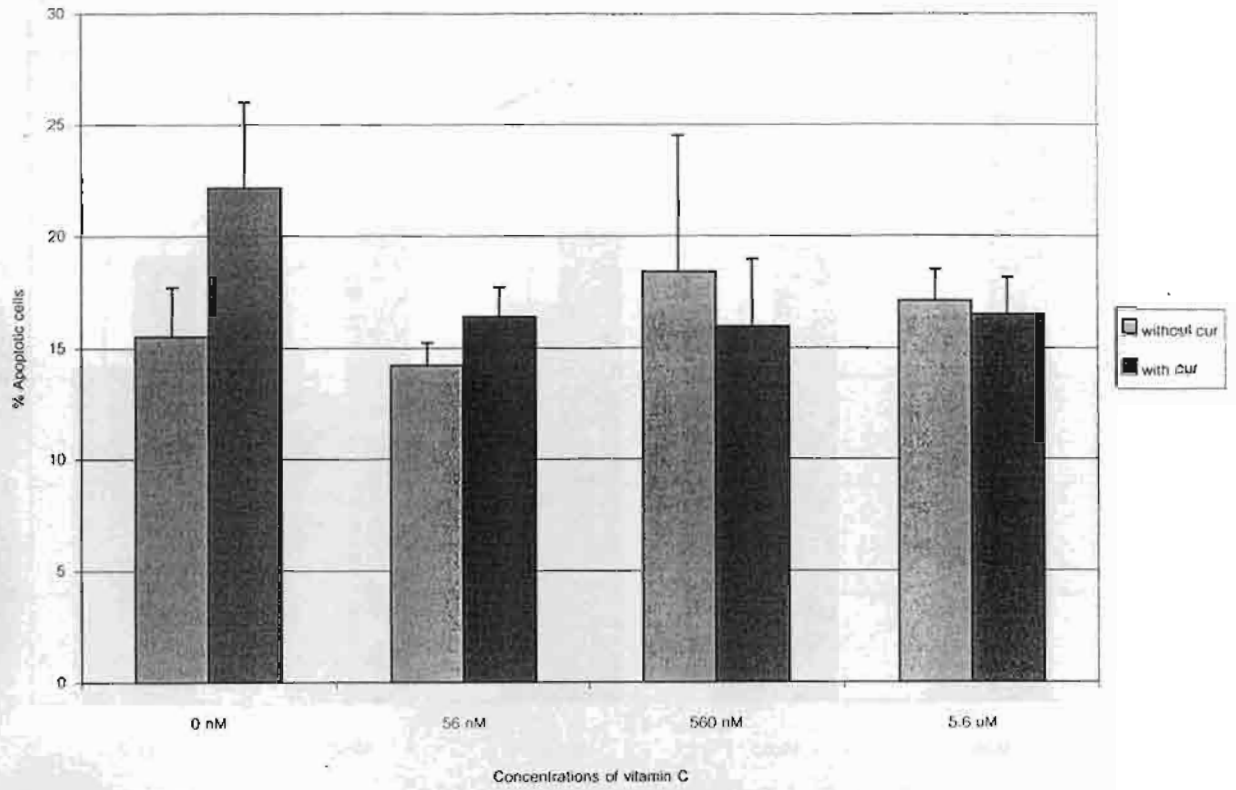


Fig. 8 The effect of vitamin C on HL-60 cell apoptosis induced by curcumin (10 μM) when treated simultaneously at various concentrations.

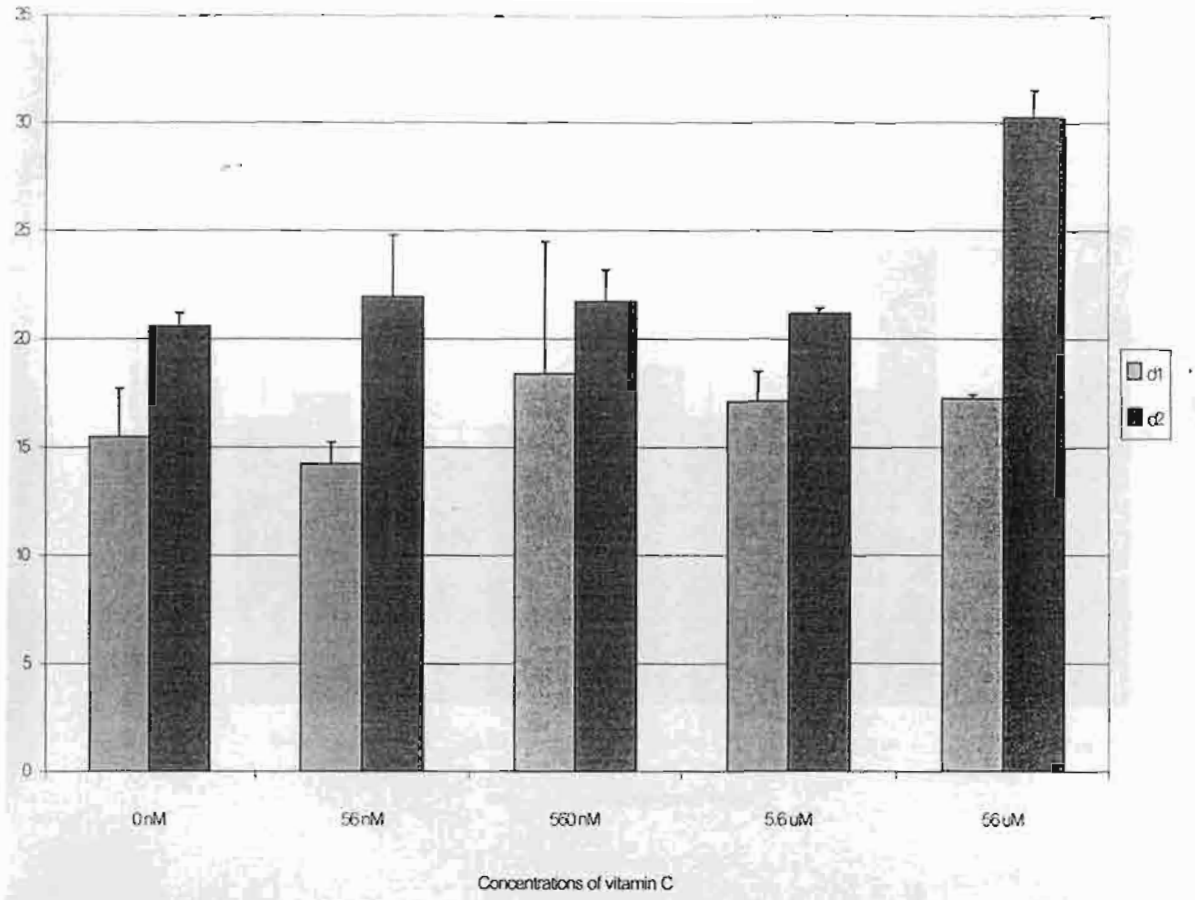


Fig. 9 The effect of vitamin C on HL-60 cell apoptosis on day 1 and day 2.

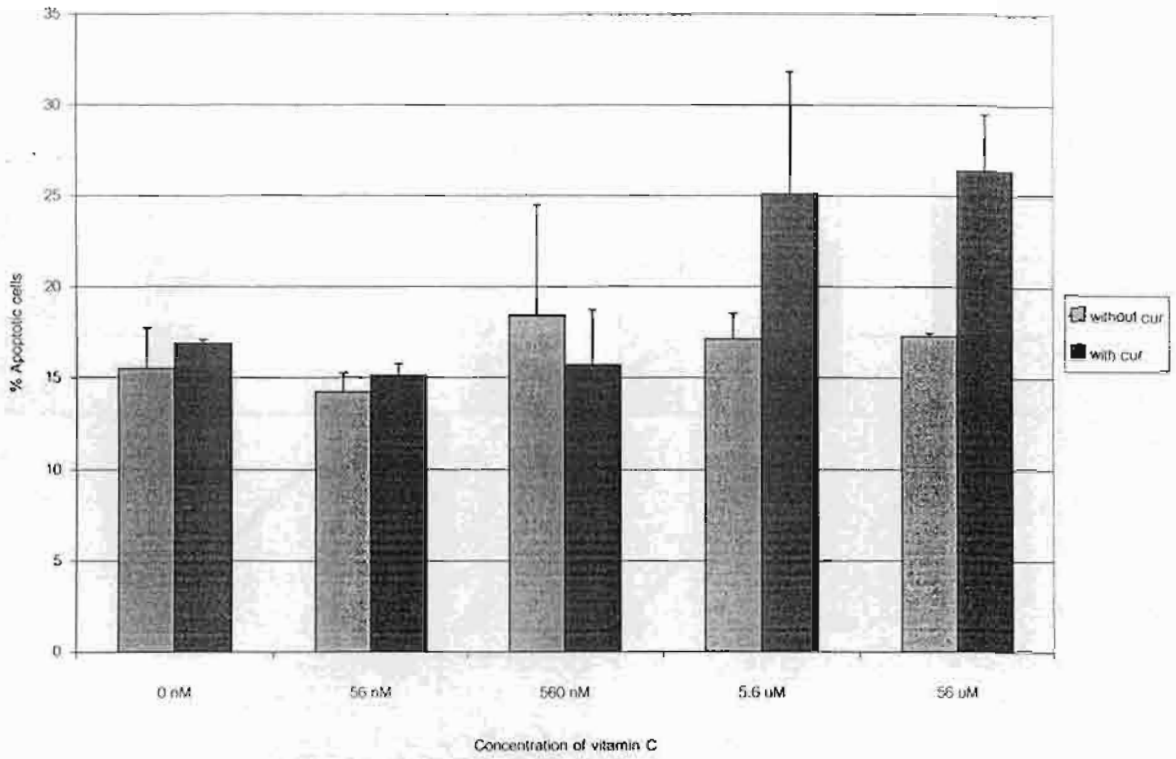


Fig. 10 The effect of vitamin C on HL-60 cell apoptosis treated for 1 day and then treated with curcumin (10 μM) for another day.

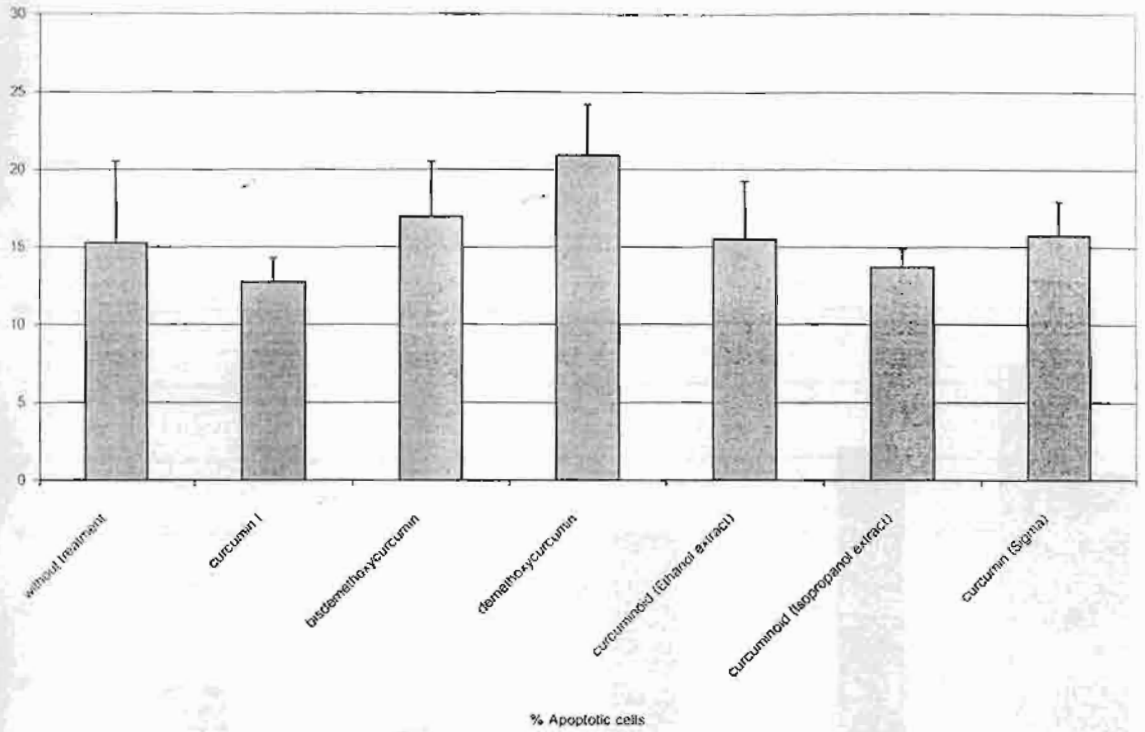


Fig. 11 The effect of curcuminoids (Ethanol extract and isopropanol extract) and curcumin derivatives on HL-60 cell apoptosis induced at the concentration of 10 μM.

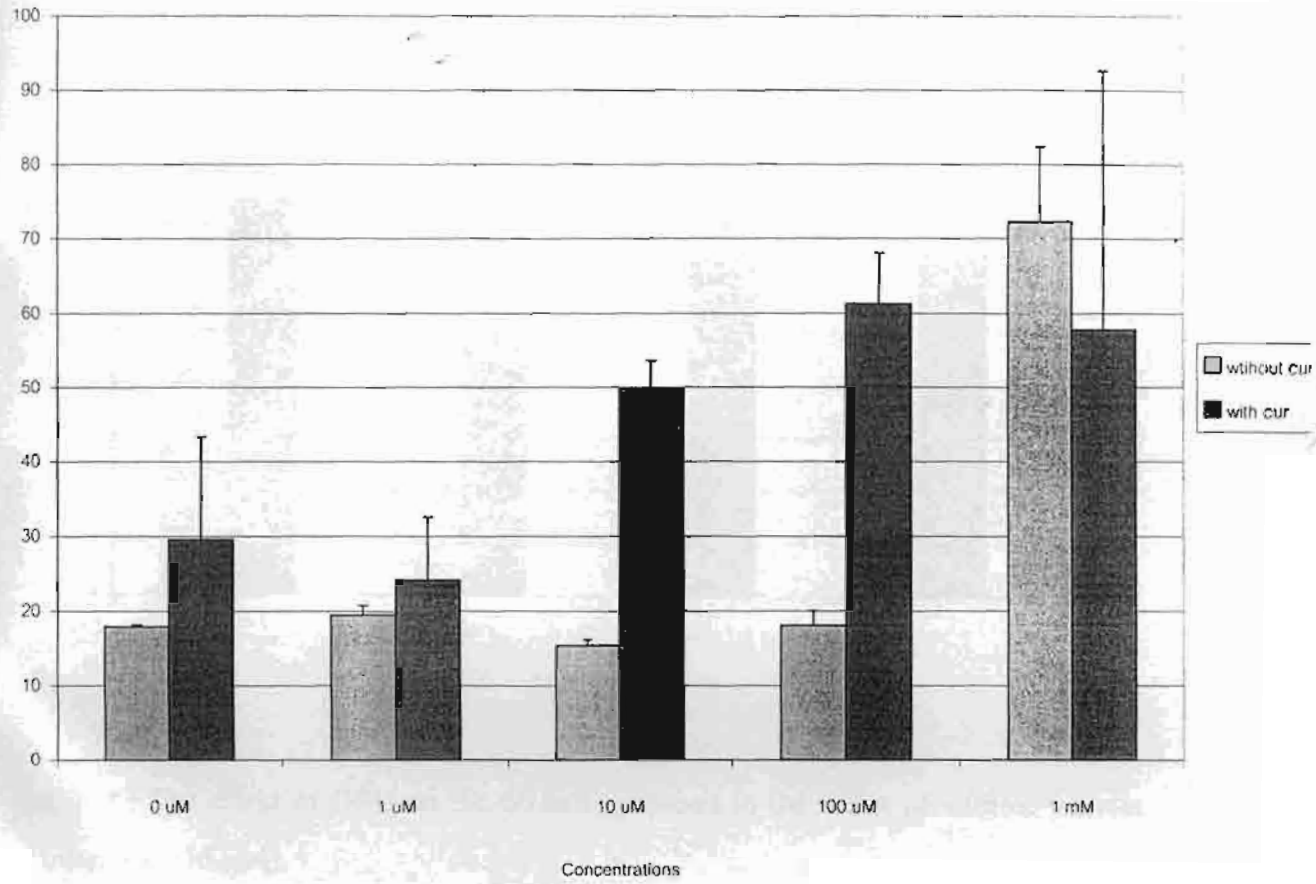


Fig. 12 The effect of Trolox on HL-60 cell apoptosis in the presence or absence of curcumin (10 μM).

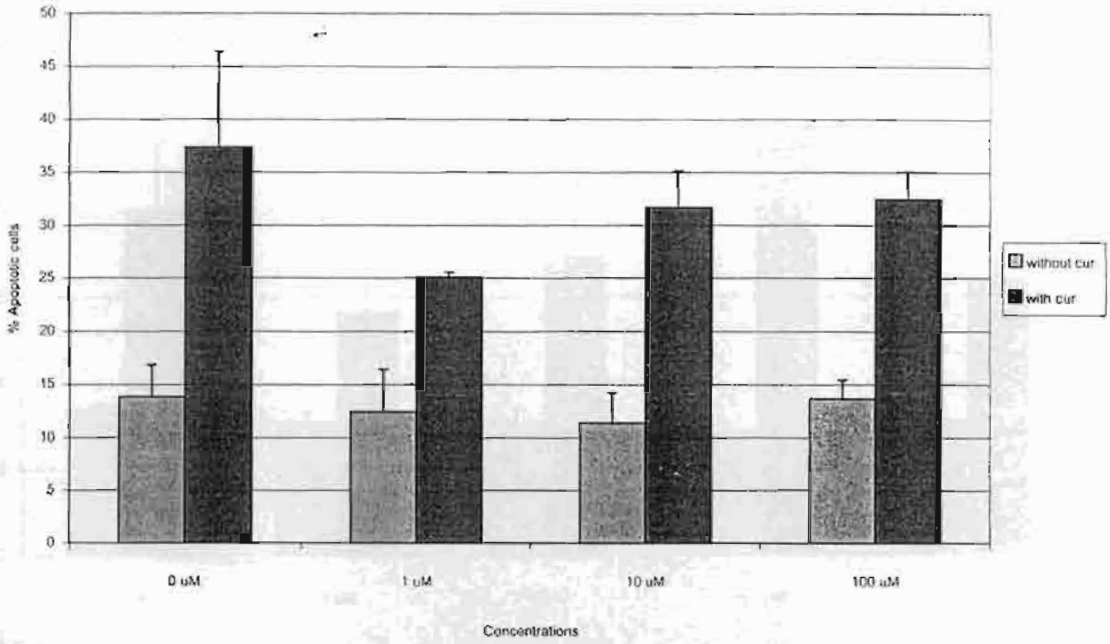


Fig. 13 The effect of GSH on HL-60 cell apoptosis in the status of with or without curcumin (10 μM).

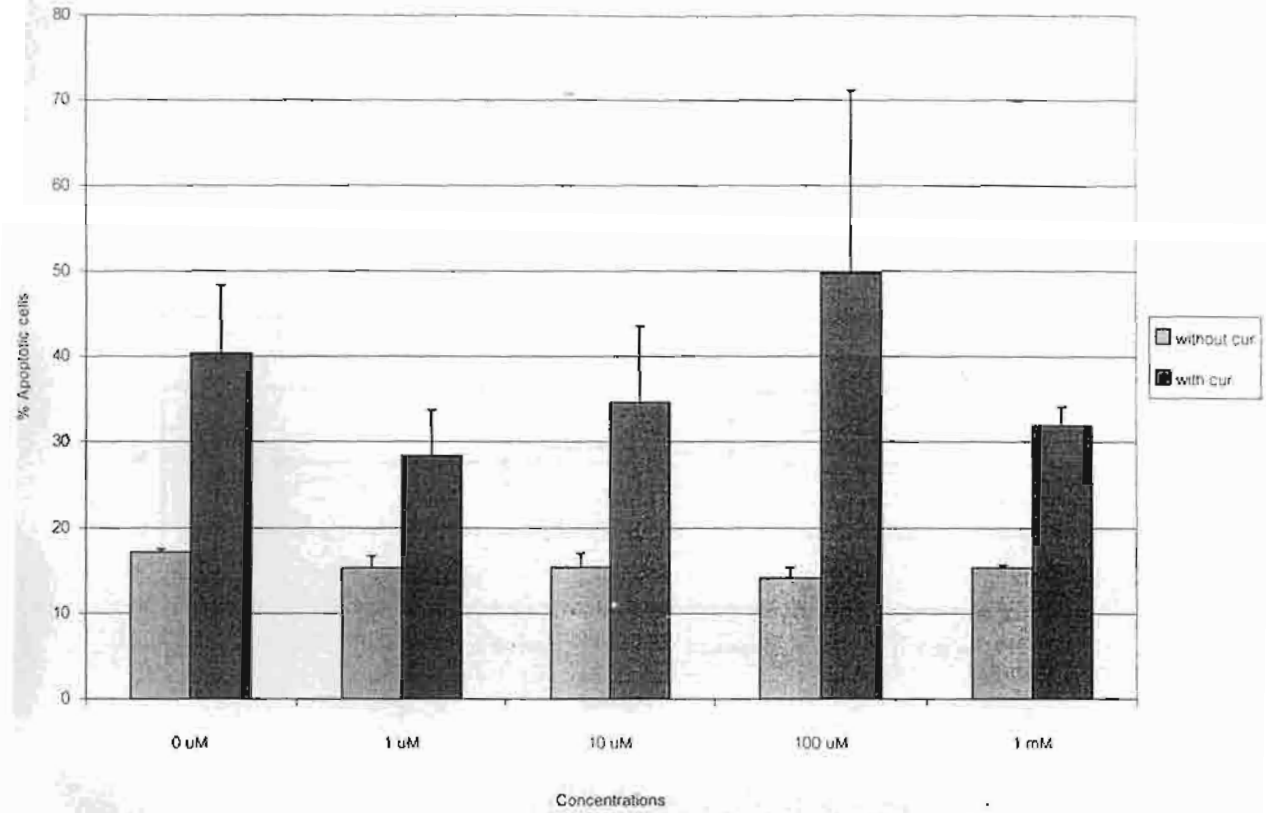


Fig. 14 The effect of N-acetylcysteine on HL-60 cell apoptosis in the status of with or without curcumin (10 μM).

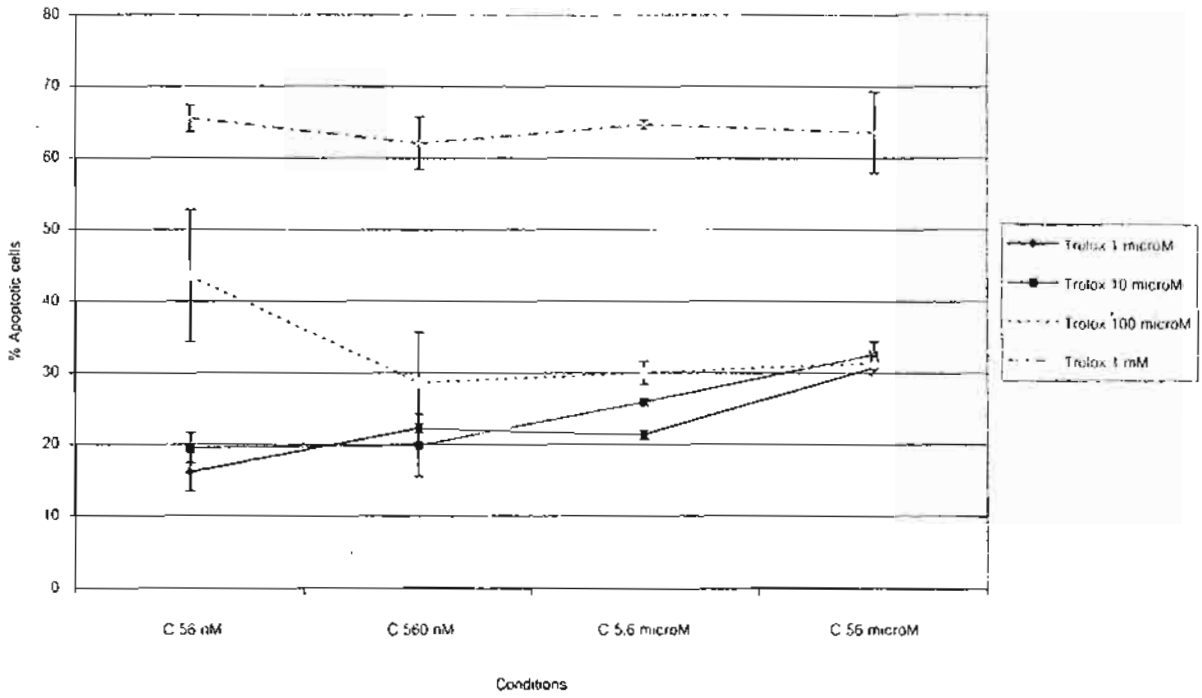


Fig. 15 The effect of vitamin C and Trolox on HL-60 cell apoptosis induced by curcumin at 10 μM.

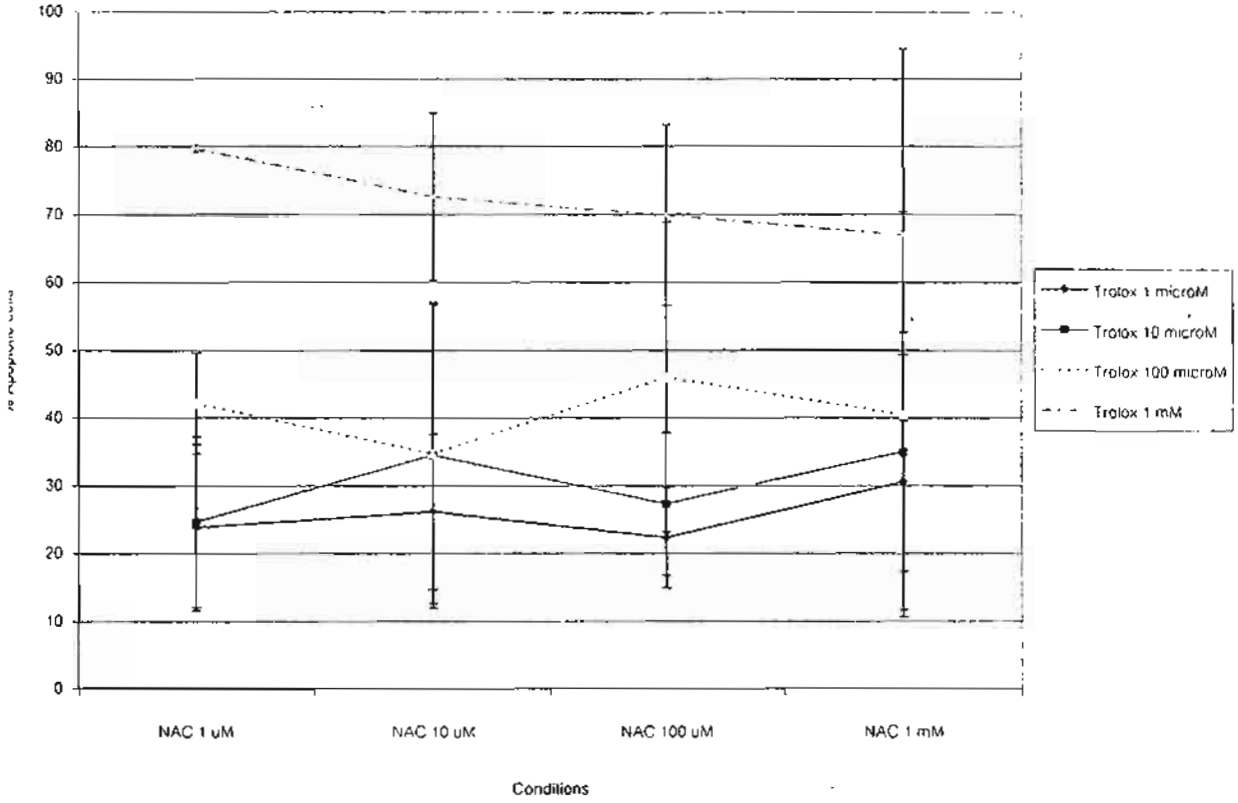


Fig. 16 The effect of Trolox and NAC on HL-60 cell apoptosis induced by curcumin at 10 μM.

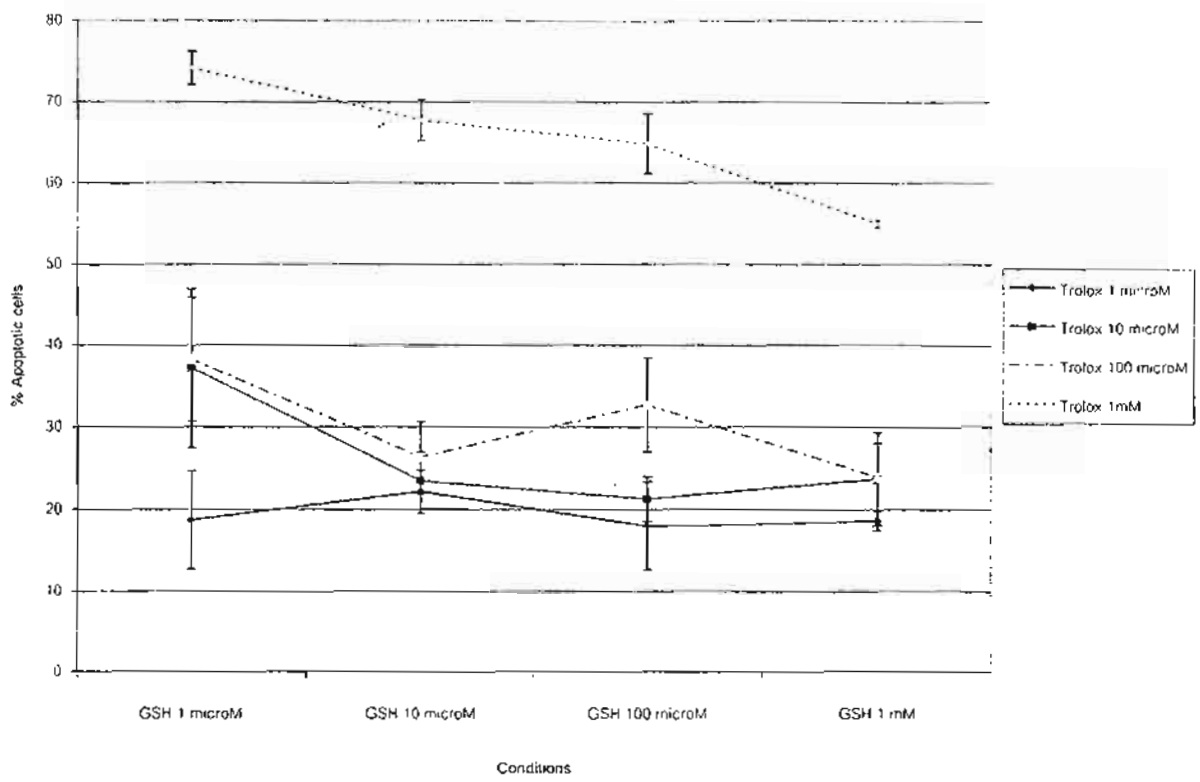


Fig. 17 The effect of Trolox and GSH on HL-60 cell apoptosis induced by curcumin at 10 μM.

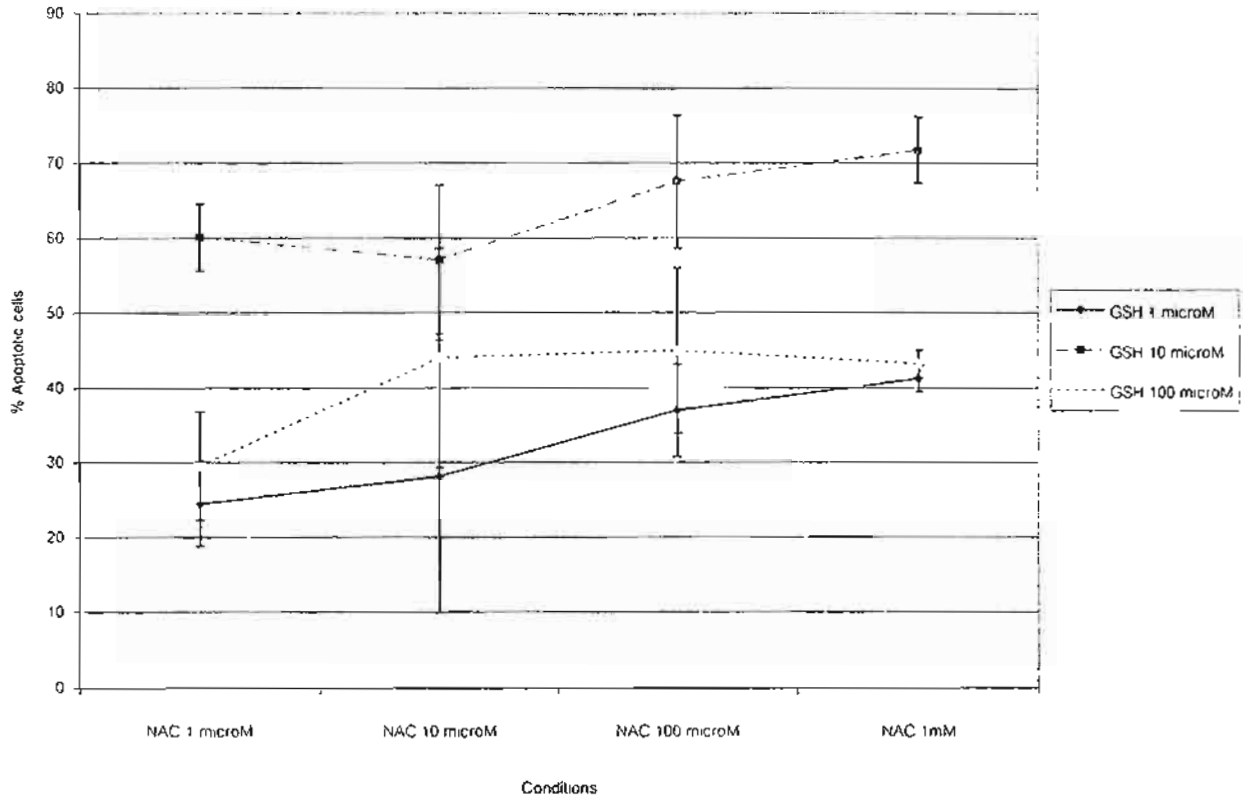


Fig. 18 The effect of GSH and NAC on HL-60 cell apoptosis induced by curcumin at 10 μM.

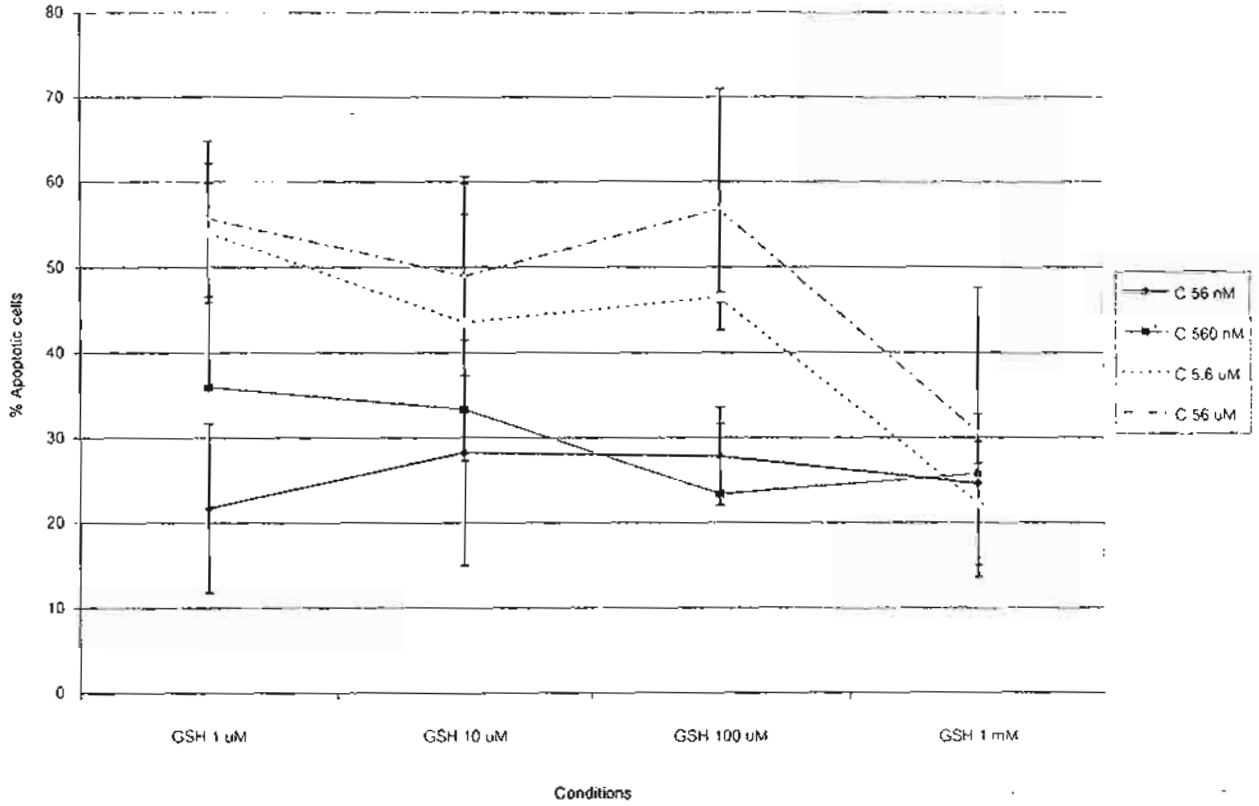


Fig. 19 The effect of vitamin C and GSH on HL-60 cell apoptosis induced by curcumin at 10 μM.

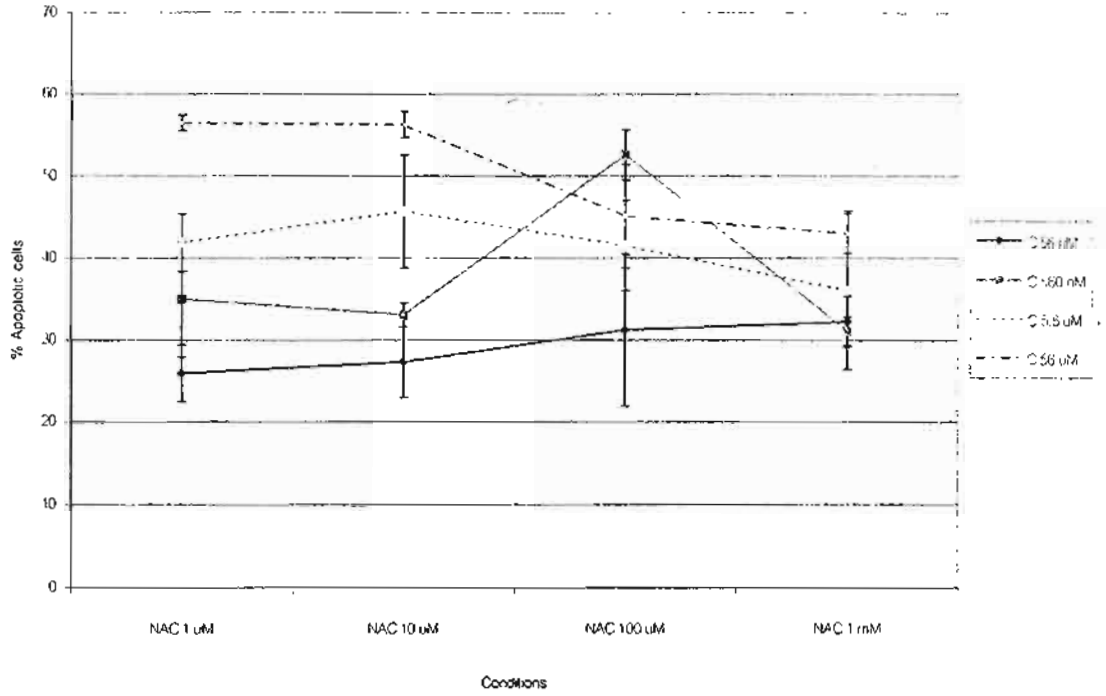


Fig. 20 The effect of vitamin C and NAC on HL-60 cell apoptosis induced by curcumin at 10 μM.

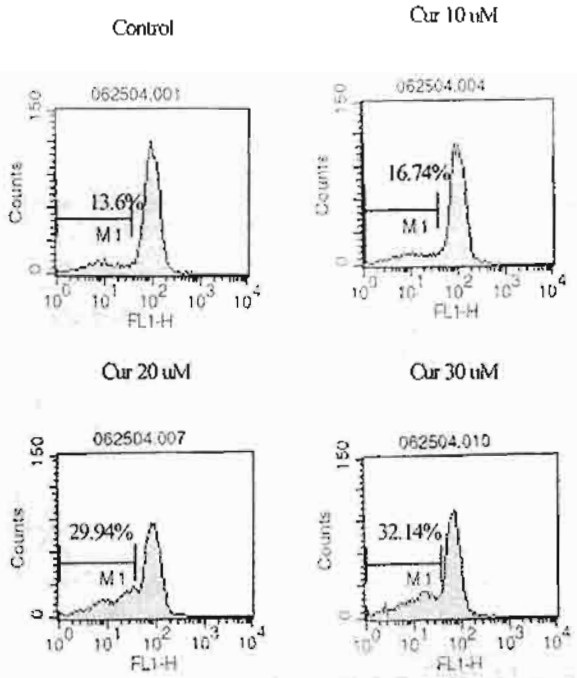


Fig. 21 Reduction of mitochondrial membrane potential when HL-60 cells were treated with curcumin at 0, 10, 20 and 30 μM for 4 h. Aliquots of $1x10^6$ cells were incubated with 50 nM 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and analyzed by flow cytometry. The percentages reflect the reduction of mitochondrial membrane potential.

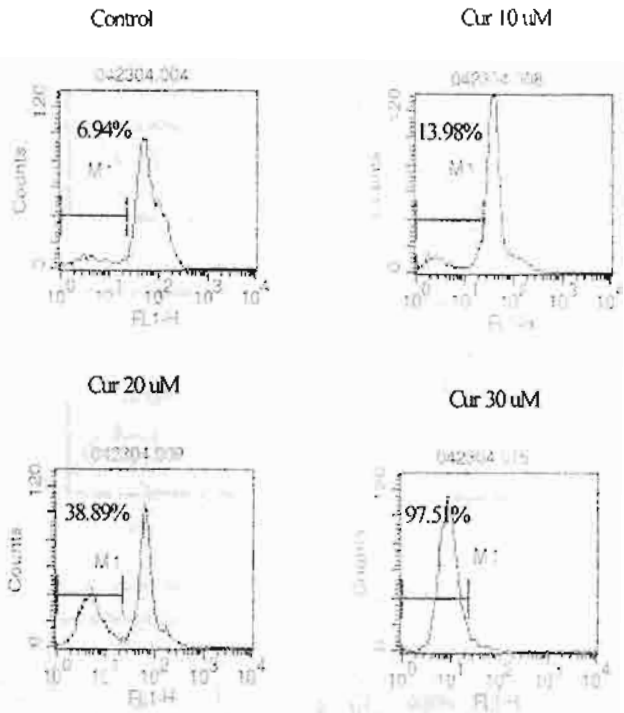


Fig. 22 Reduction of mitochondrial membrane potential when HL-60 cells were treated with curcumin at 0, 10, 20 and 30 μM for 24 h.

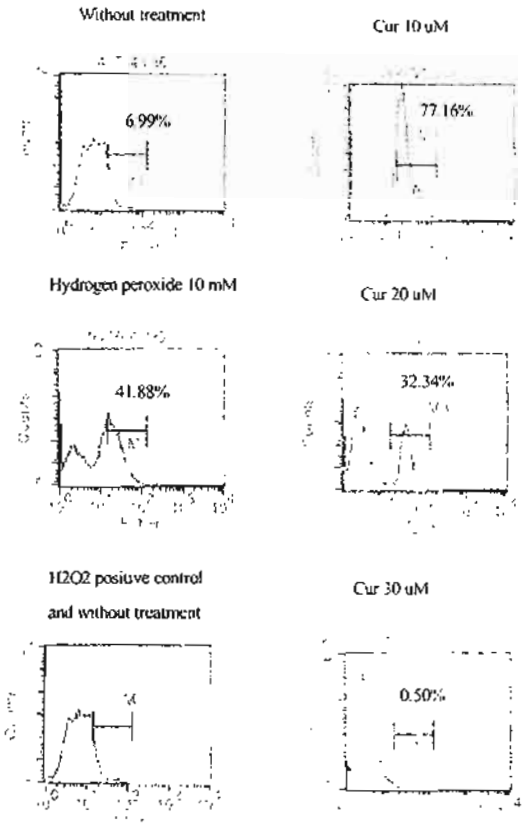


Fig. 23 The production of ROS (peroxides or DCFH) after curcumin treatment for 4 h. The open peak at left lower picture was a positive control after 10 mM hydrogen peroxide treatment (overlay).

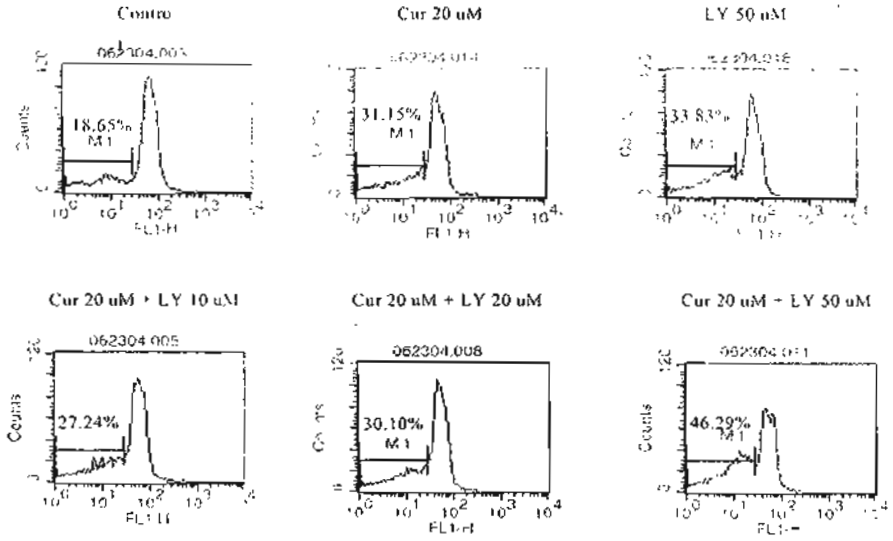


Fig. 24 The pattern of mitochondrial membrane potential when HL-60 cells were pretreated with LY294002 (PI3K inhibitor) at 10, 20 and 50 μM for 50 minutes and then with curcumin at 20 μM for 4 hours or with curcumin 20 μM alone or with LY294002 50 μM alone for 4 hours. Then $DiOC_6(3)$ was added at 40 nM for 15 minutes at 37 °C and measured mitochondrial membrane potential by using flow cytometry as mentioned in Materials and Methods.

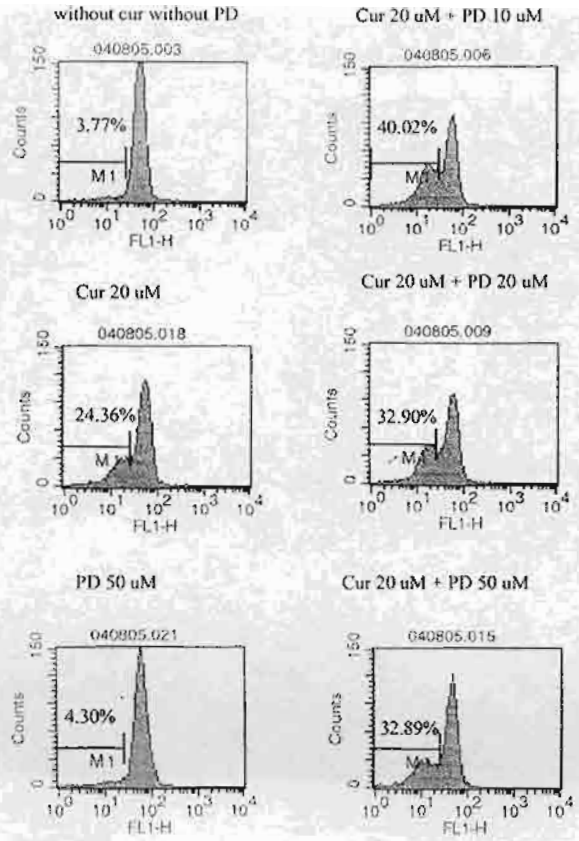


Fig. 25 The effect of PD98059 (MEK or MAP kinase kinase inhibitor) on HL-60 cells induced by curcumin. HL-60 cells were pretreated with PD98059 for 50 min and then incubated with curcumin (20 μM) for 4 hours. The HL-60 cells were treated with curcumin (20 μM) alone or PD98059 (50 μM) alone were compared. The mitochondrial membrane potential was measured as mentioned in Materials and Methods.

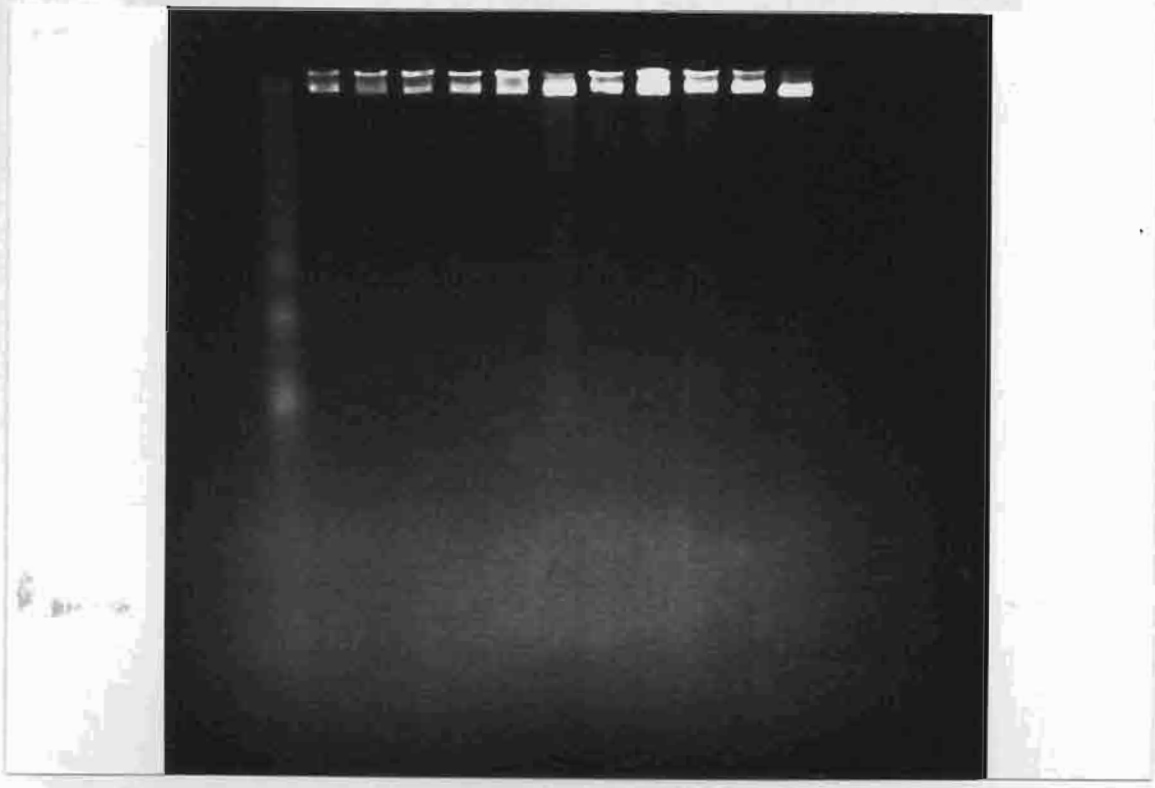


Fig. 26 Agarose gel electrophoresis pattern of HL-60 cells treated with curcumin. Lane 1 HL-60 cells treated with camtothecin 3 μM; Lane 2 control (without treatment); Lane 3 HL60 cells treated with curcumin 1 ng/ml day 1; Lane 4 with curcumin 10 ng/ml day 1; Lane 5 with curcumin 100 ng/ml day 1; Lane 6 with curcumin 1 μg/ml day 1; Lane 7 with curcumin 10 μg/ml day 1; Lane 8 without treatment day 1; Lane 9 with curcumin 1 μg/ml day 1; Lane 10 curcumin 2 μg/ml day 1; Lane 11 curcumin 4 μg/ml day 1 and Lane 12 curcumin 8 μg/ml day 1. It was in a ladder pattern only in lane 1, which was the cells treated with camtothecin (positive control). Other lanes showed negative results.

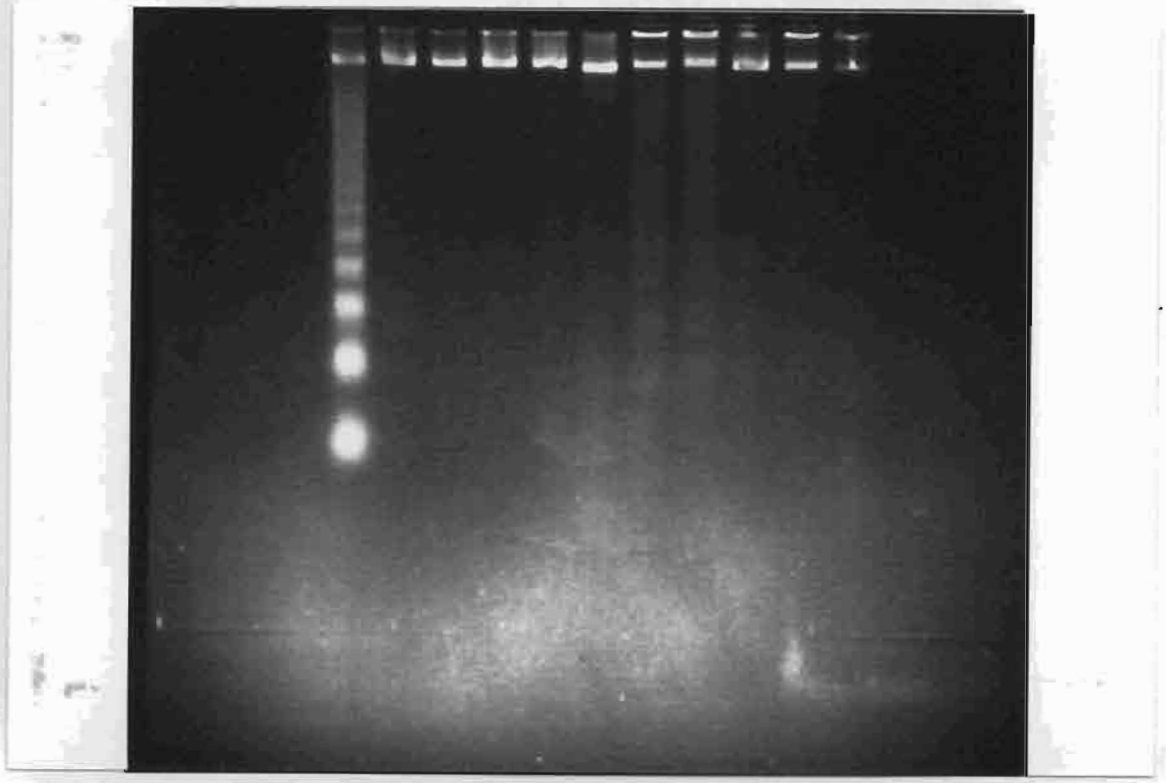


Fig. 27 Agarose gel electrophoresis pattern of HL-60 cells treated with curcumin. Lane 1 HL-60 treated with camtothecin; lane 2 control day 1; lane 3 curcumin 1 μg/ml day 1; lane 4 curcumin 2 μg/ml day 1; lane 5 curcumin 4 μg/ml day 1; lane 6 curcumin 8 μg/ml day 1; lane 7 control day 2; lane 8 curcumin 1 μg/ml day 2; lane 9 curcumin 2 μg/ml day 2; lane 10 curcumin 4 μg/ml day 2; lane 11 curcumin 8 μg/ml day 2. There was no ladder pattern except in lane 1, which was positive control.

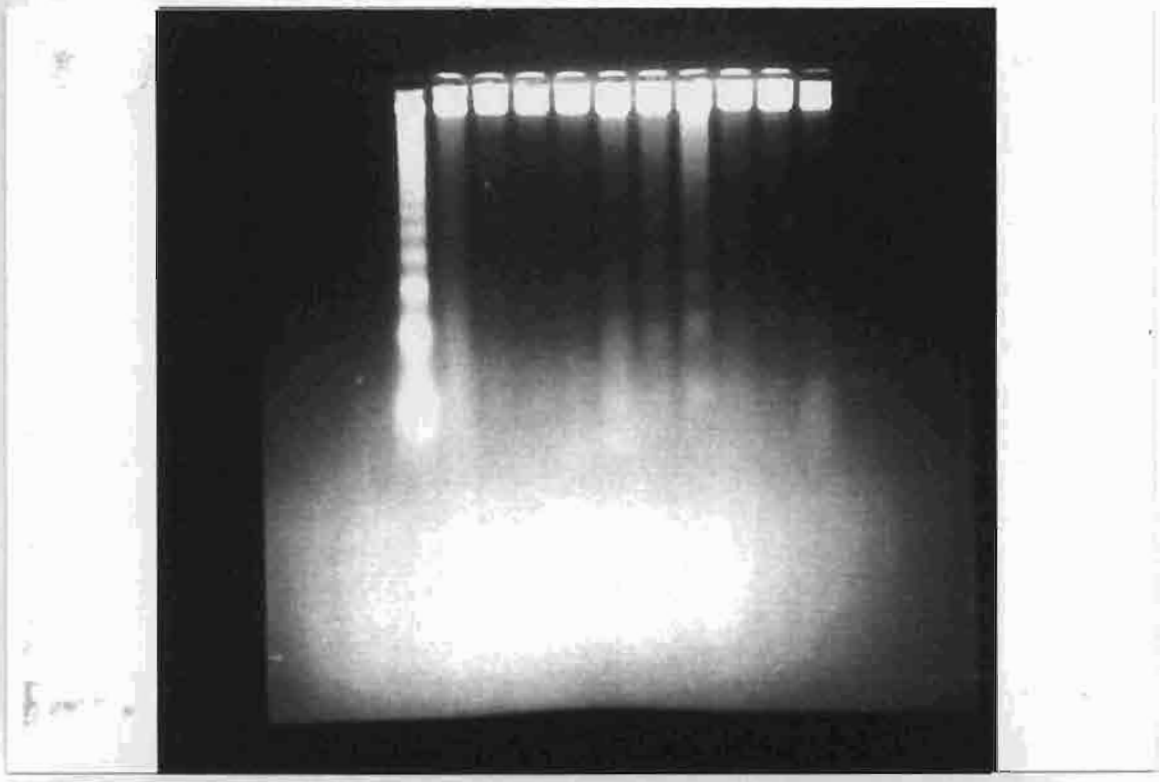


Fig. 28 Agarose gel electrophoresis pattern of HL-60 cells treated with curcumin. Lane 1 HL-60 cells treated with camtothecin; lane 2 control day 1; lane 3 curcumin 1 μg/ml day 1; lane 4 curcumin 2 μg/ml day 1; lane 5 curcumin 4 μg/ml day 1; lane 6 curcumin 8 μg/ml day 1; lane 7 control day 2; lane 8 curcumin 1 μg/ml day 2; lane 9 curcumin 2 μg/ml day 2; lane 10 curcumin 4 μg/ml day 2; lane 11 curcumin 8 μg/ml day 2. Lane 8 showed ladder pattern, which was HL-60 cells treated with curcumin 1 μg/ml (2.5 μM) day 2.

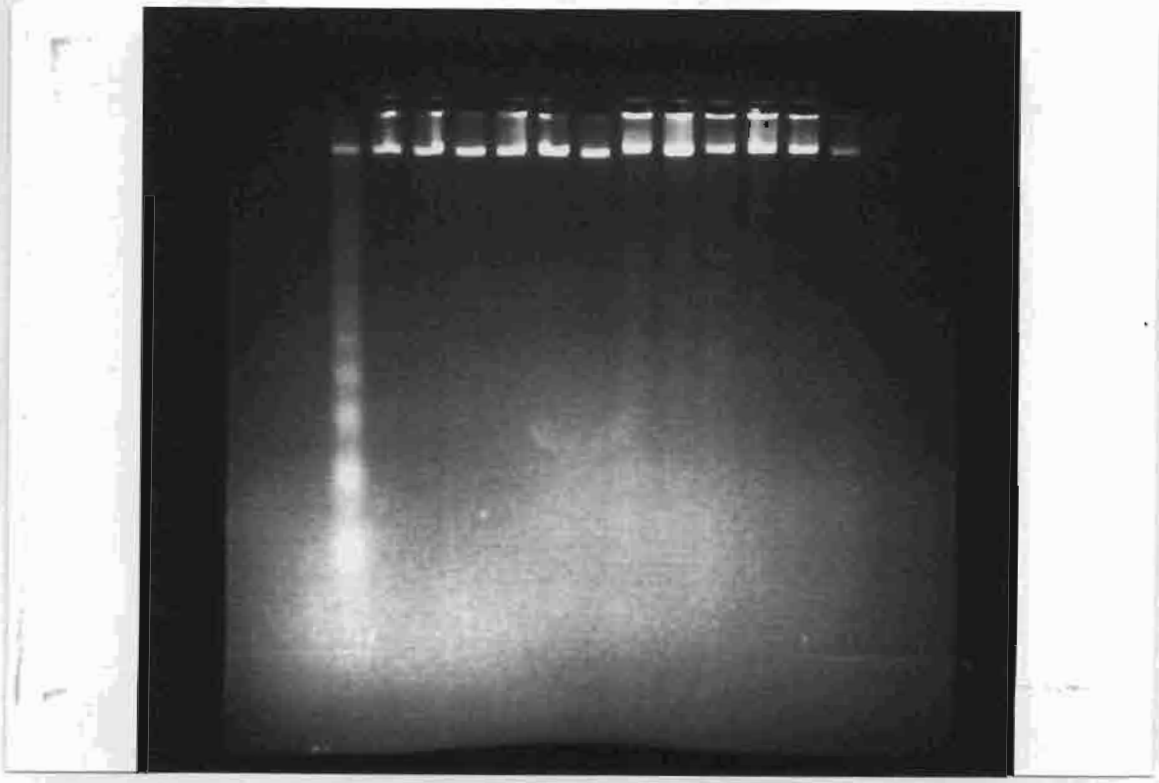


Fig. 29 Agarose gel electrophoresis pattern of HL-60 cells treated with curcuminoid extract (isopropanol) lane 1 HL-60 treated with camtothecin; lane 2 without treatment day 1; lane 3 curcuminoid (isopropanol extract) 1 ng/ml day 1; lane 4 curcuminoid (isopropanol extract) 10 ng/ml day 1; lane 5 curcuminoid (isopropanol extract) 100 ng/ml day 1; lane 6 curcuminoid (isopropanol extract) 1 µg/ml day 1; lane 7 curcuminoid (isopropanol extract) 10 µg/ml day 1; lane 8 control day 2; lane 9 curcuminoid (isopropanol extract) 1 ng/ml day 2; lane 10 curcuminoid (isopropanol extract) 10 ng/ml day 2; lane 11 curcuminoid (isopropanol extract) 100 ng/ml day 2; lane 12 curcuminoid (isopropanol extract) 1 µg/ml day 2; lane 13 curcuminoid (isopropanol extract) 10 µg/ml day 2. There was no ladder pattern in any lane except lane 1, which was positive control.

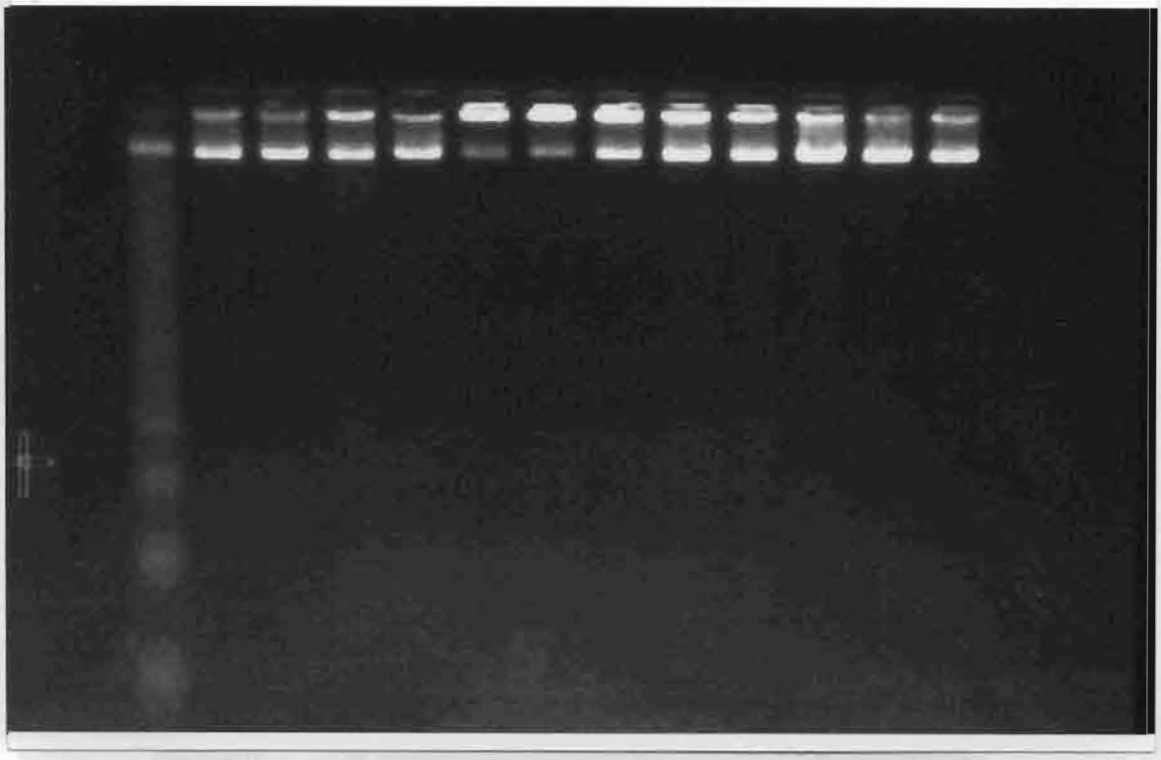


Fig. 30 Agarose gel electrophoresis pattern of HL-60 cells treated with curcuminoid (alcoholic extract). Lane 1 HL-60 cells treated with camtothecin; lane 2 control day 1; lane 3 curcuminoid (alcoholic extract) 1 ng/ml day 1; lane 4 curcuminoid (alcoholic extract) 10 ng/ml day 1; lane 5 curcuminoid (alcoholic extract) 100 ng/ml day 1; lane 6 curcuminoid (alcoholic extract) 1 µg/ml day 1; lane 7 curcuminoid (alcoholic extract) 10 µg/ml day 1; lane 8 control day 2; lane 9 curcuminoid (alcoholic extract) 1 ng/ml day 2; lane 10 curcuminoid (alcoholic extract) 10 ng/ml day 2; lane 11 curcuminoid (alcoholic extract) 100 ng/ml day 2; lane 12 curcuminoid (alcoholic extract) 1 µg/ml day 2; lane 13 curcuminoid (alcoholic extract) 10 µg/ml day 2. All showed no ladder pattern except in lane 1, which was positive control.

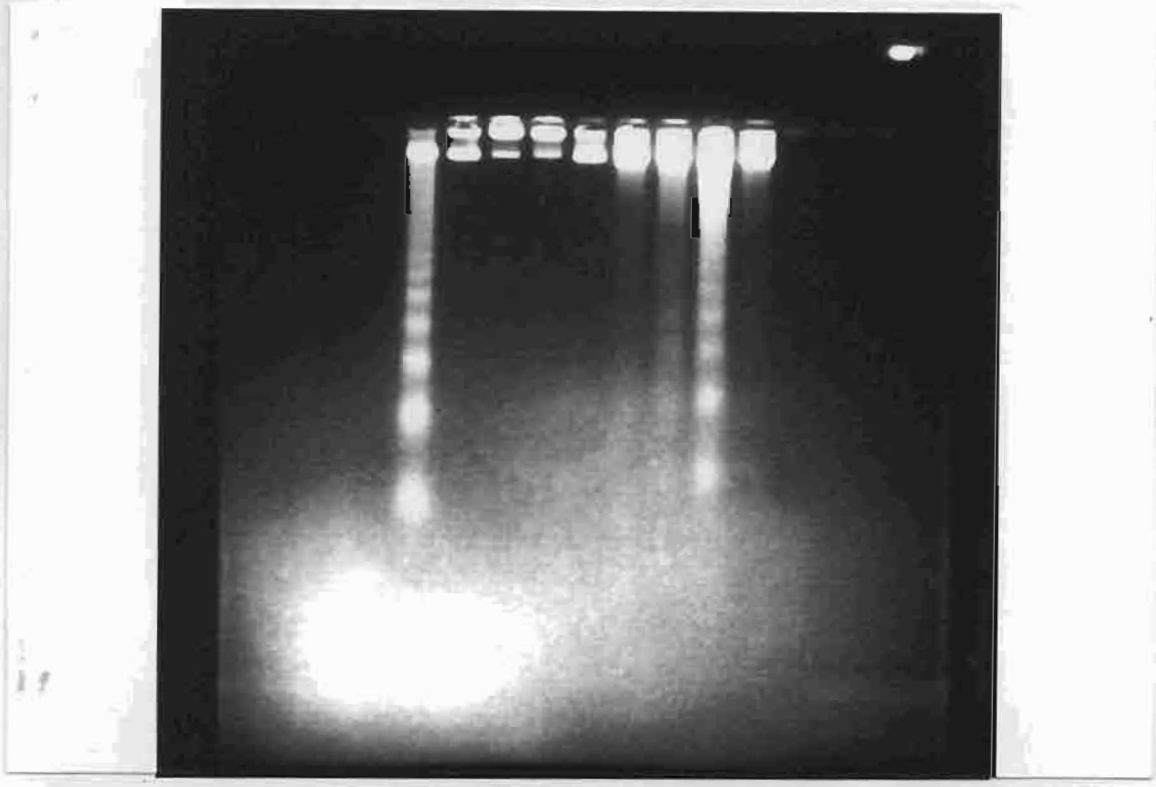


Fig. 31 Agarose gel electrophoresis pattern of HL-60 cells treated with hydrogen peroxide in media or PBS. Lane 1 HL-60 cells treated with camtothecin; lane 2 control in PBS; lane 3 HL-60 cells treated with 0.1 mM hydrogen peroxide in PBS; lane 4 HL-60 cells treated with 1 mM hydrogen peroxide in PBS; lane 5 HL-60 cells treated with 10 mM hydrogen peroxide in PBS; lane 6 control in media; lane 7 HL-60 cells treated with 0.1 mM hydrogen peroxide in media; lane 8 HL-60 cells treated with 1 mM hydrogen peroxide in media; lane 9 HL-60 cells treated with 10 mM hydrogen peroxide in media. Lane 7 and 8 (HL-60 cells treated with hydrogen peroxide 0.1 and 1 mM) showed ladder pattern, which was in the same pattern with positive control (lane 1).

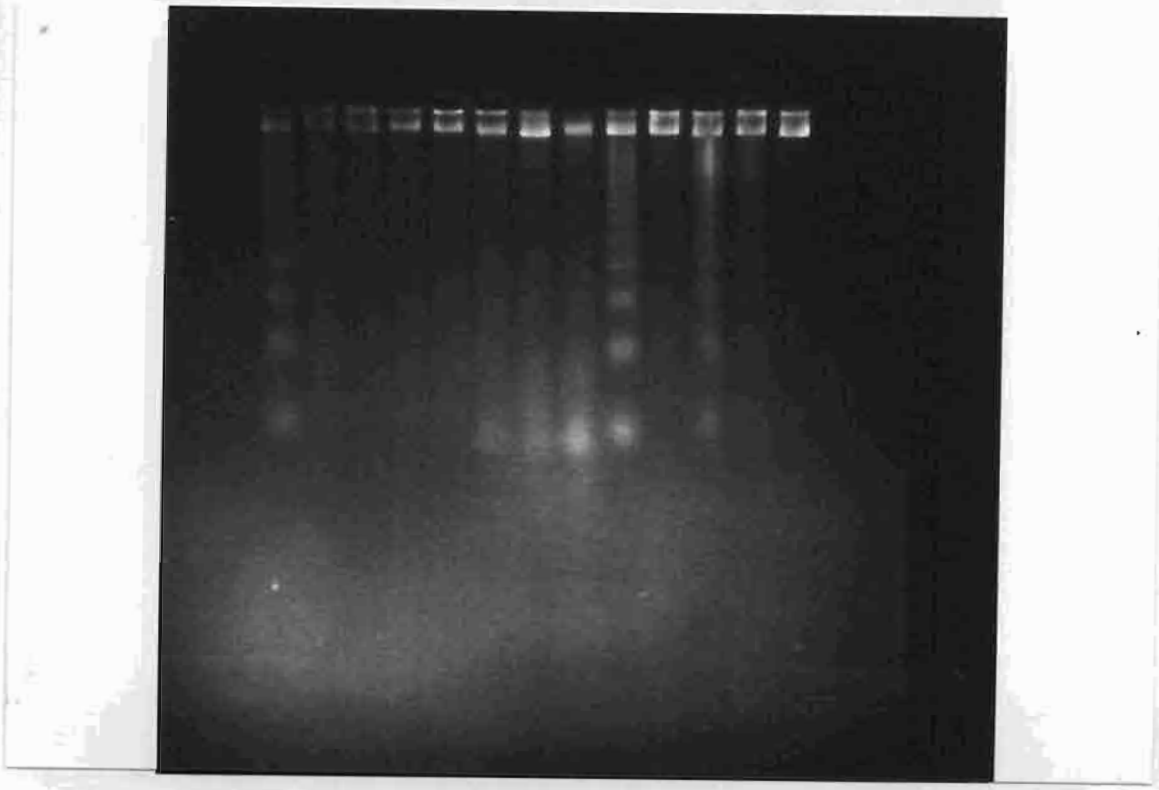


Fig. 32 Agarose gel electrophoresis pattern of HL-60 cells treated with vitamin C and curcumin simultaneously. Lane 1 HL-60 cells treated with camtothecin; lane 2 negative control; lane 3 HL-60 cells treated with vitamin C 56 nM + curcumin 10 μM; lane 4 HL-60 cells treated with vitamin C 0.56 μM + curcumin 10 μM; lane 5 HL-60 cells treated with vitamin C 5.6 μM + curcumin 10 μM. Lane 6 HL-60 cells treated with curcumin 10 μM; lane 7 HL-60 cells treated with curcumin 15 μM; lane 8 HL-60 cells treated with curcumin 30 μM. Lane 9 was HL-60 cells treated with camtothecin. Lane 10 was negative control (without treatment); lane 11 HL-60 cells treated with 0.1 mM hydrogen peroxide; lane 12 HL-60 cells treated with 1 mM hydrogen peroxide; lane 13 HL-60 cells treated with10 mM hydrogen peroxide. There was ladder pattern in lane 1 and 9, which were positive control. Lane 6-8 (HL-60 cells treated with curcumin 10, 15 and 30 μM, respectively) showed small DNA fragment which was the initial band at low molecular weight of the ladder pattern. Lane 11 also demonstrated the ladder pattern (0.1 mM hydrogen peroxide treated cells).

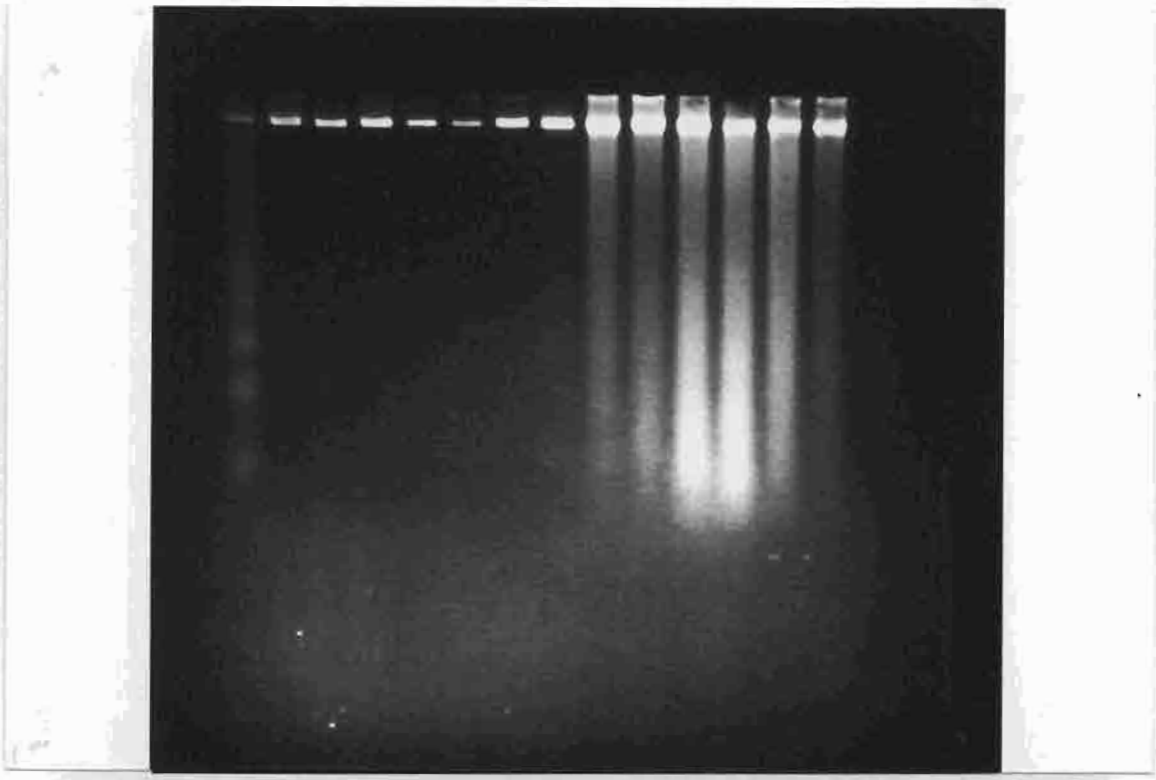


Fig. 33 Agarose gel electrophoresis pattern of HL-60 cells treated with vitamin C and curcumin simultaneously (lane 2 to lane 6) and when treated with vitamin C for 1 day and then with curcumin for another day at various concentrations of vitamin C and at 10 μM of curcumin. Lane 1 HL-60 cells treated with camtothecin; lane 2 without treatment day 1; lane 3 HL-60 cells treated with vitamin C 56 nM + curcumin 10 μM simultaneously; lane 4 HL-60 cells treated with vitamin C 0.56 μM + curcumin 10 μ M simultaneously; lane 5 HL-60 cells treated with vitamin C 5.6 μM + curcumin 10 μ M simultaneously; lane 6 HL-60 cells treated with vitamin C 56 μM + curcumin 10 μ M simultaneously; lane 7 HL-60 cells treated with curcumin 10 μM alone; lane 8 HL-60 cells treated with vitamin C 56 μM alone; lane 9 without treatment (1 day + 1 day); lane 10 HL-60 cells treated with vitamin C 56 nM + curcumin 10 μM (1 day + 1 day); lane 11 HL-60 cells treated with vitamin C 0.56 μM + curcumin 10 μM (1 day + 1 day); lane 12 HL-60 cells treated with vitamin C 5.6 μM + curcumin 10 μM (1 day + 1 day); lane 13 HL-60 cells treated with vitamin C 56 μM + curcumin 10 μM (1 day + 1 day); lane 14 curcumin 10 μM alone (treatment on day 2). In lane 10-13, it was smear pattern, which was the character of necrotic cell death.

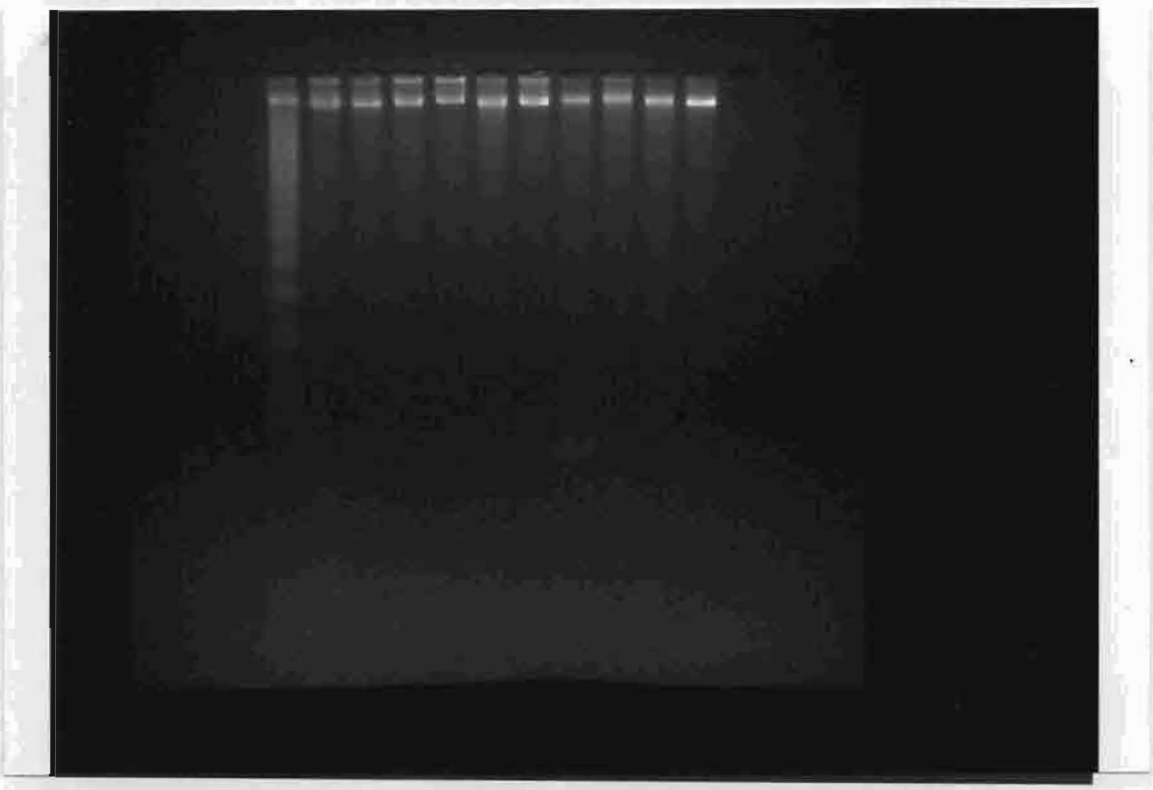


Fig. 34 Agarose gel electrophoresis pattern of HL-60 cells treated with GSH and curcumin. Lane 1 HL-60 cells treated with camtothecin; lane 2 without treatment; lane 3 HL-60 cells treated with curcumin 10 μM alone; lane 4 HL-60 cells treated with GSH 1 μM; lane 5 HL-60 cells treated with GSH 10 μM; lane 6 HL-60 cells treated with GSH 100 μM; lane 7 HL-60 cells treated with GSH 1 mM; lane 8 HL-60 cells treated with GSH 1 μM + curcumin 10 μM; lane 9 HL-60 cells treated with GSH 10 μM + curcumin 10 μM; lane 10 HL-60 cells treated with GSH 100 μM + curcumin 10 μM; lane 11 HL-60 cells treated with GSH 1 mM + curcumin 10 μM. There was no ladder pattern except in lane 1, which was positive control.

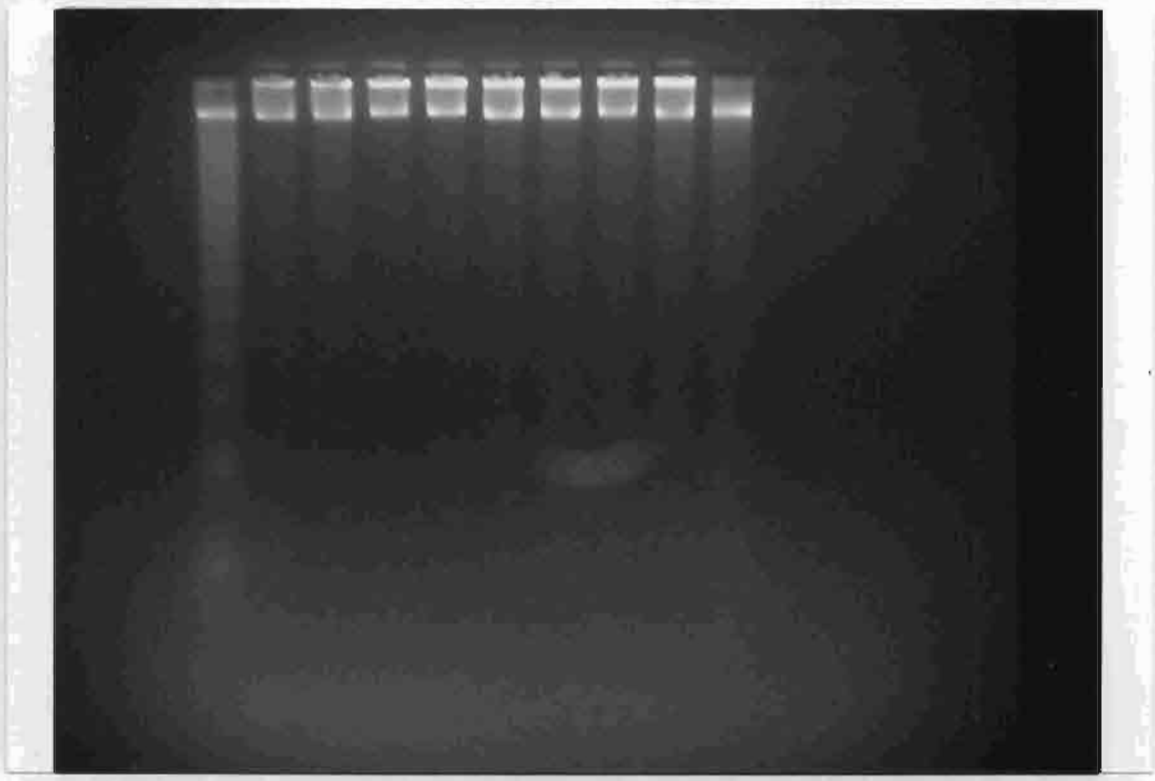


Fig. 35 Agarose gel electrophoresis pattern of HL-60 cells treated with Trolox and curcumin. Lane 1 HL-60 cells treated with camtothecin alone; lane 2 HL-60 cells without treatment; lane 3 HL-60 cells treated with curcumin 10 μM alone; lane 4 HL-60 cells treated with Trolox 10 μM; lane 5 HL-60 cells treated with Trolox 100 μM; lane 6 HL-60 cells treated with Trolox 1 mM; lane 7 HL-60 cells treated with Trolox 1 μM + curcumin 10 μM; lane 8 HL-60 cells treated with Trolox 10 μM + curcumin 10 μM; lane 9 HL-60 cells treated with Trolox 100 μM + curcumin 10 μM; lane 10 HL-60 cells treated with Trolox 1 mM + curcumin 10 μM. There was no ladder pattern except in lane 1, which was positive control.

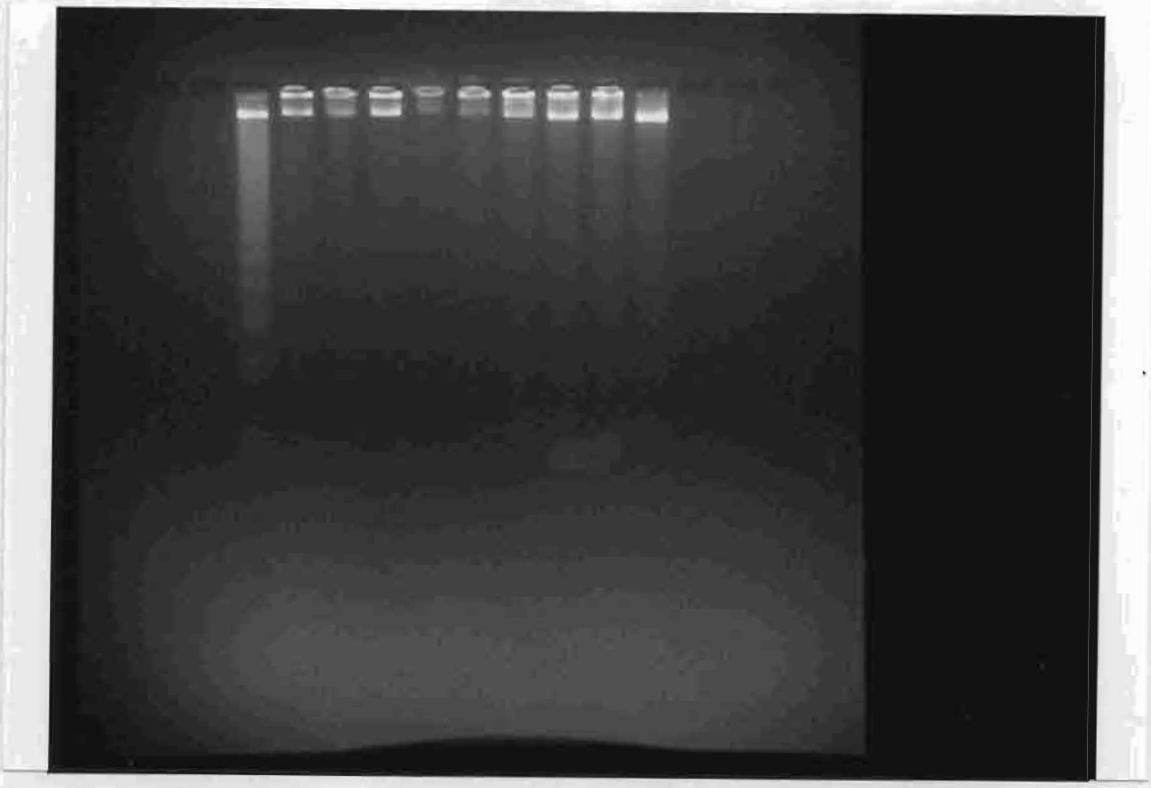


Fig. 36 Agarose gel electrophoresis pattern of HL-60 cells treated with vitamin C and curcumin. Lane 1 HL-60 cells with camtothecin; lane 2 HL-60 cells without treatment; lane 3 HL-60 cells treated with curcumin 10 μM alone; lane 4 HL-60 cells treated with vitamin C 560 nM; lane 5 HL-60 cells treated with vitamin C 5.6 μM; lane 6 HL-60 cells treated with vitamin C 56 μM; lane 7 HL-60 cells treated with vitamin C 56 nM + curcumin 10 μM; Lane 8 HL-60 cells treated with vitamin C 560 nM + curcumin 10 μM; lane 9 HL-60 cells treated with vitamin C 5.6 μM + curcumin 10 μM; lane 10 HL-60 cells treated with vitamin C 56 μM + curcumin 10 μM. There was no ladder pattern except in lane 1, which was positive control.

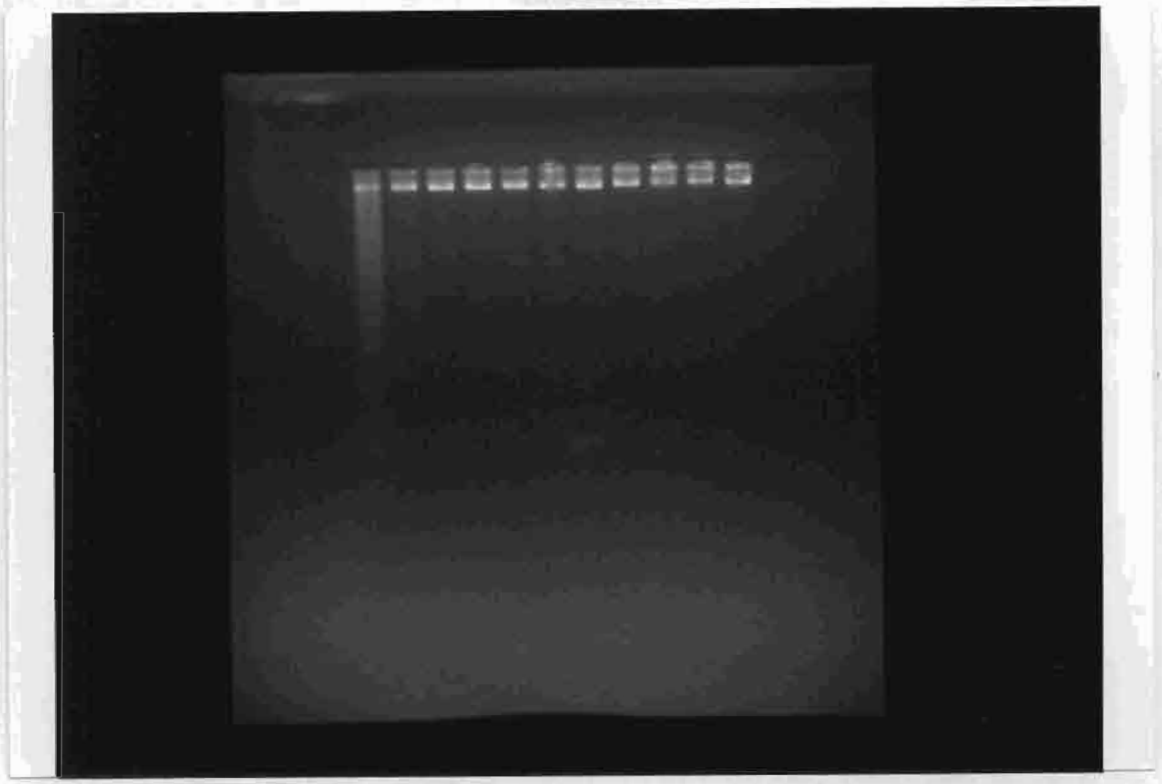


Fig. 37 Agarose gel electrophoresis pattern of HL-60 cells treated with NAC and curcumin. Lane 1 HL-60 cells treated with camtothecin; lane 2 HL-60 cells (without treatment); lane 3 HL-60 cells treated with curcumin 10 μM alone; lane 4 HL-60 cells treated with NAC 1 μM; lane 5 HL-60 cells treated with NAC 10 μM; lane 6 HL-60 cells treated with NAC 100 μM; lane 7 HL-60 cells treated with NAC 1 mM; lane 8 HL-60 cells treated with NAC 1 μM + curcumin 10 μM; lane 9 HL-60 cells treated with NAC 10 μM + curcumin 10 μM; lane 10 HL-60 cells treated with NAC 100 μM + curcumin 10 μM; lane 11 HL-60 cells treated with NAC 1 mM + curcumin 10 μM. There was no ladder pattern except in lane 1, which was positive control.

Discussion

Curcumin is the major compound of food flavoring turmeric (*Curcuma longa* Linn.), and has been used as a herbal medicine. Curcumin shows a variety of physiological effects, and several studies indicate curcumin to be anticarcinogenic (46), and anti-inflammatory (47). Curcumin further shows antioxidant properties: curcumin acts as a superoxide radical scavenger (48, 49) and as a singlet oxygen quencher (50). Of the naturally occurring curcuminoids, tetrahydrocurcumin, one of the main metabolites of curcumin exhibits the most potent antioxidative activity (51). Contrary to the antioxidant nature of curcuminoids, much evidence for cytotoxic properties of curcumin was reported, and its cytotoxicity is suggested to be due to production of reactive oxygen species. Treatment of DNA with curcumin was demonstrated to cause strand scission of DNA, and the addition of ROS scavenger BHA or BHT can prevent DNA from the curcumin-mediated strand breaks, suggesting that curcumin-mediated production of ROS may participate in the DNA damage (52, 53).

Very recently it was shown that curcumin acted as prooxidant by forming reactive oxygen species through the reduction of copper causing activation of oxygen molecule. Hydroxyl radical generated from hydrogen peroxide resulting from the dismutation of superoxide anion could break the DNA strand, and further form DNA adduct 8-OHdG. Addition of catalase prevented DNA from the strand breaks and the formation of DNA base adduct, indicating that curcumin/copper-dependent formation of hydroxyl radical car play a principle role in the DNA damage. Curcumin can act as a prooxidant by activating molecular oxygen through the reduction of transition metals in cells, resulting in the DNA damages including the formation of base adduct and induction of apoptotic cell death (54). From our experiment on agarose gel electrophoresis, when HL-60 cells were treated with hydrogen peroxide in media compared to PBS, it was found that there was ladder pattern in the system of treatment cells with hydrogen peroxide in media but not in PBS (Fig. 31). This might be because of the presence of ferrous iron in the complete media with fetal bovine serum. This metal iron involved in the generation of hydroxyl radical via the Fenton reaction. It is corresponding to the finding that free radicals could cause DNA strand break.

In HL-60 cells treated with curcumin there was oxidative stress in the cells. It was shown that there was ROS production by measuring the DCF-DA changing to be DCF in HL-60 cells after incubating with curcumin at various concentrations (10, 20, and 30 μM). There was an increase in fluorescence compared to the control (without treatment). It was in a dose response manner (54). Yoshino's work showed the effect at 0, 5, and 10 μM and did not show at higher doses. We showed that at 10 μM, the level of peroxides and hydrogen peroxides was maximal and decreased at 20, and 30 μ M (Fig. 23). This finding corresponded to the morphology of the cells when examined under fluorescence microscope after staining with propidium iodide, which was fragmented bodies or apoptotic bodies at the two higher concentrations. That caused the decreased in the intensity and amount of fluorescence when the cells were treated with higher concentrations of curcumin. Another reason is that the apoptotic cells underwent secondary necrosis.

The increased dose of curcumin caused the more decrease in mitochondrial membrane potential (Fig. 21, 22). At higher doses of curcumin, the more decreased in mitochondrial membrane potential and the higher amount of apoptotic cells were found.

Curcumin-mediated apoptosis of HL-60 cells was closely related to the increase in the concentration of reactive oxygen species in cells. Generation of reactive oxygen species was a key factor of induction of apoptosis by curcumin.

It was reported that curcumin induced cell death in HL-60 cells, both sensitive and with MDR phenotypes (caused by overexpression of P-gp, i.e., HL-60/Vinc; or MRP1, i.e., HL-60/Adr), which could be classified as caspase-3 dependent apoptosis, together with cytochrome c release, activation of caspase-3 and oligonucleosomal DNA fragmentation. No active caspase-8 was detected. This work was compared the effect of curcumin with UVC in HL-60 cells which could induced apoptosis via caspase-3 activation (55).

When HL-60 cells were treated with 25 μM curcumin for 24 h, the expression level of Mcl-1 (antiapoptotic protein) was down regulated, but that of Bax and Bak up-regulated time-dependently. There was significant difference in the expression level of Mcl-1, Bax and Bak between the curcumin-treated groups and control group ($p<0.05$-0.01). At the same time, curcumin had no effect on progress of cell cycle in primary acute myelogenous leukemia at newly diagnosis, but could increase the peak

of sub-G1 ($p<0.05$), and down-regulate the expression of Mcl-1 and up-regulate the expression of Bax and Bak with the difference being statistically significant. The expression of p27kipl, p21wafl and pRbp were elevated and that of cyclin D3 decreased in the presence of curcumin. These finding suggested that the Bcl-2 gene family indeed participated in the regulatory process of apoptosis induced by curcumin in HL-60 cells and AML cells. Curcumin can induce apoptosis of primary acute myelogenous leukemic cells and disturb cell cycle progression of HL-60 cells. The mechanism appeared to be mediated by perturbing G0/G1 phases checkpoints which associated with up regulation of p27kipl, p21wafl and pRbp expression, and down-regulation of cyclin D3 (56).

The effect of curcumin on the activation of the apoptotic pathway in human acute myelogenous leukemia HL-60 cells and in established stable cell lines expressing Bcl-2 and Bcl-xl was found. Curcumin inhibited the growth of HL-60 cells (neo) in a dose- and time-dependent manner, whereas Bcl-2 and Bcl-xl-transfected cells were relatively resistant. Curcumin activated caspase-8 and caspase-3 in HL-60 neo cells but not in Bcl-2 and Bcl-xl-transfected cells. Similarly, time dependent poly(ADP)ribose polymerase (PARP) cleavage by curcumin was observed in neo cells but not in Bcl-2 and Bcl-xl-transfected cells. Curcumin treatment also induced Bid cleavage and mitochondrial cytochrome c release in neo cells but not in Bcl-2 and Bcl-xl transfected cells. In neo HL-60 cells, curcumin also downregulated the expression of cyclooxygenase-2, Because DN-FLICE blocked curcumin-induced apoptosis, caspase-8 must play a critical role. Overall, the results indicated that curcumin induces apoptosis through mitochondrial pathway involving caspase-8, Bid cleavage, cytochrome c release, and caspase-3 activation. These results suggested that Bcl-2 and Bcl-xl are critical negative regulators of curcumin-induced apoptosis (40).

The role of AP-1 and acidosis was pivotal. Intracellular acidification caused by agents such as UV, etoposide or ceramide accompanied the progression of apoptosis. It was suggested that cellular acidosis might set favorable conditions for a dormant, low pH-dependent (acidic) nuclease, which could be involved in intranuclesomal genome degradation, a hallmark of programmed cell death. In HL-60 cells, acidic nuclease up-regulation triggered by acidic agents followed the induction of AP-1 transcription factor active complexes and accompanied the progression of

apoptosis. Inhibition of AP-1 binding to DNA and c-jun synthesis, protected cells from genome destruction (57).

In contrast, in HL-60 cells, ESR spectroscopy demonstrated that curcumin produced radicals under alkaline conditions, scavenged the superoxide anion radical, and enhanced the radical intensity of sodium ascorbate at higher concentrations. It was also found that catalase did not reduce the cytotoxic activity of curcumin (58).

It was reported that apoptosis-inducing activity of curcumin was not affected by cycloheximide, actinomycin D, EGTA, W7 (calmodulin inhibitor), and sodium orthovanadate. By contrast, an endonuclease inhibitor $ZnSO_4$ and proteinase inhibitor N-tosyl-L-lysine chlorotetradecanoylphorbol-13 acetate (TPA) had partial effect. The antioxidants, N-acetyl-L-cysteine (NAC), L-ascorbic acid, alpha-tocopherol, catalase and superoxide dismutase, all effectively prevented curcumin induced apoptosis (59). In this work, the researchers used only one concentration of each antioxidant, i.e., NAC at 5 mM, ascorbic acid at 100 μM, and alpha-tocopherol at 100 μM compared to our work which used various concentrations of each antioxidant and it had different effect. For ascorbic acid or vitamin C, it prevented the HL-60 cell apoptosis induced by curcumin at all concentrations (56, 560 nM, and 5.6 μM). Meanwhile NAC and vitamin E had dual effect. However, for vitamin E, we used the analog or derivative of vitamin E that can dissolve in water, which is different from alpha-tocopherol that is lipophilic. We also studied the combined effects of the antioxidants and demonstrate the significance of their effects by using two way ANOVA.

It was demonstrated that treatment of human leukemia HL-60 cells with ceramide, a breakdown product of sphingomyelin, induced both programmed cell death and cellular differentiation. Apoptosis in response to ceramide occurred in a concentration-dependent manner. Apoptosis induced by ceramide in HL-60 cells required the presence of c-jun protooncogene. However, apoptosis was inhibited by curcumin, a specific inhibitor of c-jun/AP-1. Whereas curcumin restored ability of inhibited cells to grow, it does not affect ceramide-induced differentiation. These results indicated that ceramide controled cell differentiation and proliferation through apoptosis by activating the nuclear transcription factor AP-1. Further, AP-1 is apparently more closely related to apoptosis-inducing signal transduction pathway than to the pathway leading to cellular differentiation (60).

The effect of antioxidants on HL-60 cell apoptosis induced by curcumin was inconclusive and required further study. In this process, there was an increase in oxidative stress as shown by the increase in fluorescence of the dye (DCFH-DA to DCF) (54). The existence of oxidative stress was confirmed that there were hydrogen peroxide and peroxide radicals production. But the effect of vitamin C, Trolox, NAC and GSH could have various effects, not just protection of the cells from apoptosis as expected since they all are antioxidants. So, the mechanism of curcumin in inducing apoptosis was complicated. Concerning the combined effect of antioxidants on HL-60 cell apoptosis induced by curcumin, it indicated that Trolox acted as prooxidant rather than antioxidant when combined with vitamin C ($p<0.05$) or combined with GSH ($p<0.05$) Fig. 15 and Fig. 17, respectively. The prooxidant effect was also observed in the combination of vitamin C and NAC ($p<0.05$) as shown in Fig. 20. However, the single effect of each antioxidant was not significantly different except GSH compared to the treatment with curcumin alone.

The apoptotic death of HL-60 cells demonstrated the appearance of fragmented DNA as shown by the band at the low molecular weight of DNA at the initial band of ladder pattern of positive control (HL-60 cells treated with camtothecin) as shown in Fig. 32. No ladder pattern was shown in HL-60 cells treated with curcumin might be because of the heterogeneity of cells, i.e. not all cells under went apoptosis. For the mechanism of apoptosis in the system, we found the decrease in mitochondrial membrane potential as depicted in Fig. 21 and Fig. 22, which was in a dose- and time-response manner.

The statistical values and significance of each antioxidant were summarized in Table 1, which were from Kruskal Wallis analysis.

Table 1 Statistical values of the effect of each antioxidant on HL-60 cell apoptosis induced by curcumin (10 μM) by Kruskal Wallis analysis

Antioxidant	p value	Significant
Vitamin C	0.261	No
Trolox	0.060	No
GSH	0.031	Yes
NAC	0.263	No

The statistical values and significance of combined antioxidants were summarized in Table 2, which were analyzed by using two way ANOVA.

Table 2 Statistical values and significance of the effect of combined antioxidants on HL-60 cell apoptosis induced by curcumin (10 μM) by two way ANOVA.

Antioxidants	p value	Significant
Vitamin C + Trolox	0.001	Yes
Trolox + NAC	0.932	No
Trolox + GSH	0.001	Yes
GSH + NAC	0.322	No
Vitamin C + GSH	0.201	No
Vitamin C + NAC	0.002	Yes

We studied the effect of LY294002 (PI3K inhibitor) and found that it enhanced the membrane potential reduction, which meant that the number of apoptotic cells was increased. The mechanism involved PI3K of apoptosis was as followed. In the presence of trophic factor, binding of trophic factors stimulates PI-3 kinase activity, leading to activation of the downstream kinase Akt, which phosphorylates Bad. Phosphorylated Bad then forms a complex with the 14-3-3 protein. With Bad sequestered in the cytosol, the antiapoptotic Bcl-2/Bak-xl proteins can inhibit the activity of Bax, thereby preventing the release of cytochrome c and activation of the caspase cascade (61). In the presence of LY294002, it inhibited PI3K and induced caspases that caused apoptosis, thus it proved that PI3K is in the signal transduction cascade of HL-60 cell apoptosis induced by curcumin (Fig. 24).

On the contrary, the effect of MEK in the signaling pathway was considered directly. Since the curcumin-induced apoptosis was not inhibited by MEK inhibitor (PD98059) (Fig. 25), it meant that the apoptosis was not via this mediator but through the PI3K. It was reported that mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase (PI3K/PKB) pathway were relevant to the induction of apoptosis by curcumin in breast cell lines (20). This was not via the same mediators (PI3K and MAPK) when compared to breast cell lines (even though it was the same stimulant, i.e., curcumin). Thus, it depends on the types of cells as well. This was the

first report of the roles of antioxidants and PI3K on HL-60 cell apoptosis induced by curcumin, which the cytotoxicity of curcumin was prevented and delayed by GSH and PI3K inhibitor. The combined roles of Trolox plus vitamin C, Trolox plus GSH and vitamin C plus NAC were significantly different on the curcumin-induced apoptosis of HL-60 cells.

Compared to the concurrent report (54) as mentioned above, we found that in the system of curcumin-induced apoptosis of HL-60 cells, the oxidative stress existed. Hydrogen peroxide and peroxide radicals were detected by the change of DCFH-DA (nonfluorescence) to be DCF (fluorescence) when there were free radicals in the system as shown in Fig. 23. At 10 μM of curcumin, the amount of ROS was highest that was consistent with Yoshino's work, but in ours the reduction of ROS production at 20 and 30 μM was found. The reduction of ROS amount corresponded to the morphology of the cells, which showed apoptotic bodies or fragmented bodies (data not shown) and the appearance of fragmented DNA as shown in Fig. 32.

In the experiment of curcumin and curcumin derivative: curcumin I, demethoxycurcumin, and bisdemethoxycurcumin were compared. It was found that demethoxycurcumin was the most potent form and caused highest percentage of apoptotic cells among the others at the same concentration of 10 μM (Fig. 11). It is intriguing to further investigate the antioxidant effect on HL-60 cell apoptosis induced by each derivative and the mechanism of death of each compound whether it is the same with that of curcumin (diferuloylmethane) or not. Should antioxidants and curcumin be used in leukemic patients requires further *in vivo* experiments.

It was demonstrated that curcumin was a specific inhibitor for c-jun/AP-1 transcription factor (57). Usually there was a cross-talk in the signal transduction pathway which was also shown in HL-60 cells treated with curcumin. The present work showed that curcumin-induced apoptosis was via PI3K and curcumin itself from the previous work curcumin acted as AP-1 inhibitor. Thus, the signaling pathway in the system was complicated and needs further investigation to clarify its mechanism.

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The effect of antioxidants on curcumin-induced apoptosis in HL-60 cells

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Abstract

Curcumin is the main biologically active phytochemical compound in turmeric. Curcumin has anti-inflammatory, antioxidant, anticarcinogenic and antiproliferative activities. The present work was aimed to determine the effects of vitamin C, Trolox, reduced glutathione (GSH) and *N*-acetylcysteine (NAC) on human promyelocytic leukemic (HL-60) cell apoptosis induced by curcumin and its mechanism of cell death. HL-60 cells were incubated with curcumin for 24 h and apoptotic cells were quantitated by flow cytometry following staining with annexin V-FITC and propidium iodide. Curcumin induced apoptosis, which was confirmed by the decrease in mitochondrial membrane potential. In the presence of 10 μM curcumin, vitamin C (56, 560 nM and 5.6 μM) and GSH (1, 10 and 100 μM) reduced the number of apoptotic cells, but NAC and Trolox had a dual effect, being protective at 1, 10 μM, and 1 mM for NAC and 1 μM, for Trolox; and synergistic at 100 μM for NAC and 10, 100 μM, and 1 mM for Trolox. The prooxidant effect was observed when Trolox was combined with vitamin C

or GSH ($p<0.05$). The effect of vitamin C together with NAC also caused HL-60 cells to die more ($p<0.05$).

Keywords: Apoptosis; Curcumin; HL-60 cells; Antioxidants.

Abbreviations: HL-60 cells, human promyelocytic leukemic cells; GSH, glutathione; NAC, N-acetylcysteine; PI3K, phosphotidylinositol 3-kinase; MAPKK, mitogen activated protein kinase kinase.

Introduction

Apoptosis is a physiological suicide mechanism that controls homeostasis, in which cell death naturally occurs during tissue turnover (Wyllie et al., 1980). It is the most common form of eukaryotic cell death. Cells undergoing apoptosis display profound structural changes, including plasma membrane blebbing and nuclear disintegration. The nuclear change is associated with extensive damage to chromatin and DNA cleavage into nucleosomal-length DNA fragments after activation of calcium-dependent endogenous endonucleases (Arends et al., 1990). Apoptosis is essential in many physiological processes, including the embryonic development and the maturation of immune system (Sanderson, 1981). It is currently the subject of intense research, partially because tumor cells are susceptible to death by apoptosis in response to drugs and/or radiation treatment.

Curcuma longa Linn. is a perennial herb originally cultivated widely in tropical regions of Asia from which dried rhizome is isolated the spice turmeric. It belongs to the family *Zingiberaceae.* Turmeric, a powder from the dried rhizomes, is used for medicinal purposes and is reportedly used as an antiseptic, a cure for poisoning, to eliminate body waste products, for treating dyspepsia, and respiratory disorders, as a

cure for some skin diseases, including wound healing, and as a house hold remedy for treating sprains and swellings caused by injury (Ammon and Wahl, 1996).

Curcumin, also known as diferuloylmethane (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), is the major yellow pigment extracted from turmeric, which is used extensively in curries. Its properties as a coloring and flavoring agent have led to uses as a dietary additive in variety of foods (Ammon and Wahl, 1996; Lin et al., 2000). Extracts containing curcumin have also been used in medicines in India and Southeast Asia for generations, and according to tradition are useful in the treatment of inflammation, skin wounds, hepatic and biliary disorders, cough, as well as certain tumors (Commandeur and Vermeulen, 1996). Curcumin has been identified to possess antimicrobial, anticancer, anti-inflammatory and topoisomerase-inhibitory activities, and has been reported to be an antioxidant and free-radical scavenger (Kuttan et al., 1985; Srimal and Dhawan, 1973; Sharma, 1976; Toda et al., 1985). As a result, dietary intake of curcumin is especially high in Asia, where adults consume up to >200 mg of curcumin/day or up to 7-8 μmol/kg of body weight. Even in France, where curcumin exposure may be more representative of that typical worldwide, intake of as much as >3.4 μmol/kg/day has been documented (Verger et al., 1998).

Several reports document an antiproliferative effect of curcumin on cultured cells such as on colon cancer and breast cancer cells (Van Erk et al., 2004; Squires et al., 2003). This may, in part, be because programmed cell death at high concentrations of curcumin can induce apoptosis such as in human leukemia cells. Reactive oxygen species (ROS) have been considered to play an important role in drug-induced apoptosis (Davis et al., 2001), thus one might suspect that curcumin, as an antioxidant,

free radical scavenger, would inhibit the ability of chemotherapeutic drugs to induce apoptosis.

Recently it has also been suggested that production of reactive oxygen intermediates may be a cause of tumor cell apoptosis as a result of curcumin treatment (Bhaumik et al., 1999). Curcumin also has been reported to induce mitochondrial abnormalities promote p53-dependent apoptosis and activation of caspase-8 and caspase-3 in human prostate cancer cell lines, HL-60 cells, human basal cell carcinoma cells, and human melanoma cells (Mukhopadhyay et al., 2001; Anto et al., 2002; Jee et al., 1998; Bush et al., 2001).

In this work, we aimed to demonstrate the effect of four antioxidant molecules, i.e., ascorbic acid, glutathione (GSH), N-acetylcysteine (NAC) and Trolox (water soluble vitamin E), on HL-60 cell apoptosis and to elucidate the combined effect of these antioxidants. Moreover, since many researchers studied the mechanisms of cucumin-induced apoptosis in HL-60 cells and reported the significance of mitochondrial leakage of cytochrome c. It motivated us to study whether the mitochondrial membrane potential changed and to identify the roles of PI3K and MEK/MAPKK in the apoptotic cell death in HL-60 cells induced by curcumin. While we were working on this research, there was a concurrent report that curcumin mediated apoptosis was related to the increase in intracellular reactive oxygen species (Yoshino et al., 2004). However, our work demonstrated that the amount of ROS was reduced at the concentrations of curcumin at 20 and 30 μM whereas in that report they measured the amount of ROS at the maximal concentration at 10 μM only which was found to be high. It meant that at higher concentrations, ROS was found in the fragmented bodies or apoptotic bodies, which were corresponded to the morphology of

the cells under treatment with curcumin at higher concentrations. Alternatively, it might be due to secondary necrosis that occurred after the process of apoptosis.

Materials and Methods

RPMI-1640 and fetal bovine serum were obtained from Gibco-BRL, New York, NY, USA. Annexin V-FITC kit was obtained from Roche, Indianapolis, IN, USA. Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), glutathione, N-acetylcysteine, vitamin C, 2',7'-dichlorofluorescein diacetate (DCFH-DA), and 3,3'-dihexyloxacarbocyanine iodide or $DiO_6(3)$ were obtained from Sigma, St. Louis, MD, USA. Trolox was obtained from Aldrich, Milw, WI, USA. LY294002 and PD98059 were obtained from Calbiochem, La Jolla, CA, USA.

Cell culture

HL-60 cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 μg/ml) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The preconfluent (growth phase) cells were treated with curcumin at concentrations as indicated for 4 or 24 hours. Diluted curcumin solutions were prepared in alcohol (0.5% final concentration). It was found that 0.5% alcohol did not affect cell viability. After incubating the cells with curcumin, the cells were processed through agarose gel electrophoresis or flow cytometry.

Treatment conditions

Preconfluent HL-60 cells ($1x10^6$ cells) were treated with hydrogen peroxide (0.1, 1, 10 mM) for 30 min at 37 °C under an atmosphere of 5% CO_2. Control cells

were incubated in the absence of hydrogen peroxide. At the termination of the incubation, catalase was added (final concentration of 100 units/ml). Cell morphology was examined under phase contrast microscope and fluorescence microscope (with propidium iodide staining). HL-60 cells were also collected for the agarose gel electrophoresis and flow cytometry.

In the conditions of treatment with the antioxidant(s): vitamin C or Trolox or N-acetylcysteine or glutathione was added simultaneously with curcumin. The concentration of vitamin C was varied from 56 nM, 560 nM, 5.6 μM, to 56 μM. For Trolox, the concentration was varied from 1 μM, 10 μM, 100 μM, to 1 mM. For N-acetylcysteine, the concentration was 1, 10, 100 μM and 1 mM. For glutathione, the dose was 1, 10, 100 μM and 1 mM. Then two components of antioxidants were added in the cell culture system to see their combined effect on HL-60 cell apoptosis induced by curcumin (at 10 μM). Then the cells were processed by using flow cytometry technique.

HL-60 cells were pretreated with LY294002 or PD98059 at 10, 20 and 50 μM for 50 min and then with curcumin at 20 μM for 4 h or with curcumin (20 μM) alone or with LY294002 (50 μM) alone or PD98059 (50 μM) alone for 4 hours. Then $DiOC_6(3)$ were added at 40 nM for 15 minutes at 37 °C and was measured for mitochondrial membrane potential.

Flow cytometry (Shapiro, 1995)

HL-60 cells were collected at the concentration of 10^6 cells. The cells were washed once and then centrifuged at 200 x g to get the cell pellets which were

resuspended in 100 μl of the binding buffer provided by the reagent kit. Annexin V-FITC (2 μl) and propidium (2 μl) were added in each tube. Then they were incubated at room temperature for 15 min in the dark. Finally the binding buffer, 900 μl, were put into it and mixed for further processing.

Mitochondrial membrane potential and reactive oxygen species (ROS) measurement (Xiang et al., 1996)

For mitochondrial membrane potential ($\Delta\Psi$) and intracellular ROS measurement, $5x10^5$ cells were incubated for 15 minutes at 37 °C with 3,3'-dihexyloxacarbocyanine iodide [$DiO_6(3)$, 40 nM], or 2',7'-dichlorofluorescein diacetate (DCFH-DA, 5 μM) followed by FACScan (Becton Dickinson) analysis.

Agarose gel electrophoresis (Gorzyca et al., 1993)

After HL-60 cells were treated with curcumin or the antioxidants, the cells were collected and centrifuged down at 200 x to yield the cell pellets. The precipitated cells were used for further process. The cells were lysed by using 0.25% Igepal in TBE (Tris-borate EDTA buffer). Then ribonuclease A (RNase A) was added to the final concentration of 1 μg/ml, incubated at 37 °C for 30 min. After that, the proteinase K was put into the lysate to the concentration of 0.1 mg/ml, and incubated at 37 °C for further 30 min. The loading dye buffer was finally added to the mixture of cell lysate and was then applied to 2 % agarose gel. The electrophoresis was processed in the condition of 60 volts for 4 h, then the gel was stained with ethidium bromide for 15 min and destained with distilled water for 30 min. Finally the DNA cleavage band or ladder pattern was observed under UV transilluminator.

If the bands were so smeared due to the ribonucleic acid (RNA), the smear was got rid off by incubating with the RNase (20 mg/ml) in TE (tris-EDTA) buffer overnight.

Statistical Analysis

The duplicate tests were performed independently in 3 experiments and analyzed based on Kruskal Wallis analysis (one way ANOVA). For the two variables the data were analyzed by using two way ANOVA.

Results

The effect of antioxidants on HL-60 cell apoptosis

When the HL-60 cells were treated with vitamin C at various concentrations, it was found that vitamin C did not cause the cells to die more than control. Then the cells were treated with vitamin C simultaneously with curcumin, it reduced the apoptotic cells at doses of 56, 560 nM and 5.6 μM. Thus, vitamin C had protective effect on apoptotic cell death as shown in Fig. 1A, but it was statistically non-significant ($p>0.05$ by Kruskal Wallis analysis).

When the cells were treated with vitamin C for 1 and 2 days, it was demonstrated that the percentages of apoptotic cells were increased at all concentrations of vitamin C (56, 560 nM, 5.6 and 56 μM) on day 2. This meant that vitamin C was not able to inhibit the apoptosis on day 2 (Fig. 1B). The number of apoptotic cells on day 1 and day 2 was not different significantly at various concentrations of vitamin C ($p>0.05$ by two way ANOVA).

When the HL-60 cells were treated with vitamin C first for 1 day and then with curcumin for another day, it was found that the percentage of apoptotic cells was increased when compared to treatment with vitamin C alone except at 56 nM vitamin C (Fig. 1C; gray bars). It meant that the treatment of vitamin C first for a day could not prevent cells from apoptosis, but it increased the number of apoptotic cells. The pretreatment of vitamin C had a synergistic effect on apoptosis when induced with curcumin except at the concentration of vitamin C at 56 and 560 nM which could reduce the number of cell death compared to treatment with curcumin alone (Fig. 1C; black bars). Hence, at these two concentrations, it inhibited HL-60 cell apoptosis. However, it was not significantly different ($p>0.05$).

Another antioxidant compound that was used to test in the experiment was Trolox which is a water-soluble vitamin E. It was found that when the HL-60 cells were treated with Trolox alone, it did not cause cells to die more at doses of 1, 10, and 100 μM but at concentration of 1 mM it caused the cells to undergo apoptosis 4 folds compared to control (without any treatment). However, in the status of treatment with curcumin 10 μM, it was found that Trolox caused the cells to undergo apoptosis more than control (curcumin 10 μM alone) at 10 μM, 100 μM, and 1 mM; whereas at the concentration of 1 μM it reduced the number of apoptotic cells. This meant that Trolox had dual effect. It was a prooxidant at 10 and 100 μM and 1 mM and showed the inhibitory effect of HL-60 cell apoptosis at 1 μM when compared to the number of apoptotic cells treated with curcumin alone as shown in Fig. 1D. However, it was not significantly different ($p>0.05$).

Glutathione (GSH), an antioxidant found mainly in the red blood cells and also in other mammal cells, was used to test the inhibitory or stimulatory effect on HL-60

cell apoptosis induced by curcumin. It was found that GSH could reduce the number of apoptotic cells compared to the system when treated with curcumin alone as shown in Fig. 1E. It was statistically significant ($p<0.05$) when analyzed by Kruskal Wallis analysis. When considering the effect of GSH alone on HL-60 cell apoptosis, it could reduce the number of apoptotic cells at 1, 10, and 100 μM compared to control.

The effect of N-acetylcysteine on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in Fig. 1F. NAC had dual effect, i.e., at concentrations of 1, 10 μM and 1 mM, it reduced the number of cell death compared to that treated with curcumin alone. Meanwhile at concentration of 100 μM, NAC enhanced the number of apoptotic cells, i.e., it had synergistic effect with curcumin in inducing apoptosis in HL-60 cells. However, it was not significantly different ($p>0.05$). When considering the effect of NAC alone (without curcumin treatment), it could decrease the number of apoptotic cells at 1, 10, 100 μM and 1 mM.

The statistical values and significance of each antioxidant were summarized in Table 1, which were from Kruskal Wallis analysis.

The combined effect of antioxidants on HL-60 cell apoptosis

The combined effect of vitamin C and Trolox on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in Fig. 2A. It was found that at 1 mM of Trolox , it enhanced the number of apoptotic cells significantly compared with that at concentration of Trolox at 1, 10, and 100 μM. When analyzed by using two way ANOVA, it was shown that the number of apoptotic cells was significantly different in various concentrations of Trolox and at different concentrations of vitamin C ($p<0.05$).

The effect of Trolox and NAC on HL-60 cell apoptosis induced by curcumin (10 μM) was demonstrated in Fig. 2B. At the concentration of Trolox at 1 mM it showed the synergistic effect to cause HL-60 cells to die more than the other concentrations of Trolox when combined with NAC. That meant at 1 mM of Trolox it acted as a potent prooxidant. However, it was not statistically different ($p>0.05$).

The effect of Trolox and GSH on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in 2C. The pattern of changes in apoptotic cells was similar to the previous ones, i.e., at 1 mM of Trolox, it enhanced the number of apoptotic cells significantly and could be concluded that at this concentration, it is a prooxidant. It was statistically different ($p<0.05$).

For the effect of GSH and NAC on HL-60 cell apoptosis induced by curcumin, it was found that at GSH 10 μM had the highest effect in enhancing the percentage of apoptotic cells, the second potent prooxidant was at 100 μM of GSH and the least potent concentration was at 1 μM as shown in Fig. 2D. It was not statistically different ($p>0.05$).

The effect of vitamin C and GSH on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in Fig. 2E. It was found that at the concentration of vitamin C at 56 μM, it could increase the percentage of apoptotic cells whereas the second potent concentration of vitamin C was at 5.6 μM, which enhanced the number of apoptotic cells but less than the concentration at 56 μM. However, it was not statistically significant ($p>0.05$).

The combined effect of vitamin C and NAC on HL-60 cell apoptosis induced by curcumin 10 mM was shown in Fig. 1F. At doses of NAC 1 and 10 μM and at concentration of vitamin C at 5.6 and 56 μM, HL-60 cells underwent apoptosis more

than other concentrations but at vitamin C of 560 nM and NAC at 100 μM had the high percentage of apoptotic cells as well. So, the number of apoptotic cells depended on the concentration of both vitamin C and NAC. The relationship of different concentrations of vitamin C and NAC on HL-60 cell apoptosis was significantly different ($p<0.05$).

The statistical values and significance of combined antioxidants were summarized in Table 2, which were analyzed by using two way ANOVA.

Gel electrophoresis

There was no ladder pattern when the HL-60 cells were treated with antioxidants (vitamin C, Trolox, NAC or GSH) at various concentrations alone or combined with curcumin at 10 μM (data not shown). HL-60 cells treated with curcumin 10, 15 and 30 μM, showed small DNA fragment which was the initial band at low molecular weight of the ladder pattern as shown in lane 6, 7, and 8, respectively (Fig. 3).

The mechanism of HL-60 cell apoptosis induced by curcumin

A reduction of mitochondrial membrane potential was noted within 4 hours of curcumin induction as shown in Fig. 4. Upper left picture showed the membrane potential of mitochondria in the HL-60 cells without curcumin treatment. When curcumin was added to the system at 10, 20, and 30 μM, there was a decrease in membrane potential in a dose response manner. Furthermore, when the incubation time with curcumin was increased to 24 hours, it was found that the decrease in

membrane potential was more than at 4 hours, which was in a time dependent manner as shown in Fig. 5 at 20 and 30 μM of curcumin treatment.

When HL-60 cells were treated with 50 μM of LY294002, a PI3K inhibitor, for 50 min, before curcumin (20 μM) treatment for 4 h. The reduction in mitochondrial membrane potential was up to 46.29% which meant that it increased the HL-60 cell apoptosis compared to that treated with curcumin (20 μM) alone (31.15%) as shown in Fig. 6.

Finally when the HL-60 cells were treated with PD98059 (a MEK or MAP kinase kinase inhibitor), it caused more in the reduction of mitochondrial membrane potential when compared to the system that treated with curcumin (20 μM) alone as shown in Fig. 7. When treating the cells with PD98059 (10 μM) and then with curcumin (20 μM),the mitochondrial membrane potential reduction was 40.02%, at 20 μM of PD it was 32.90%, and at PD 50 μM its reduction was 32.89%. It seemed that at low concentration of PD it could cause or enhance the reduction more than higher concentrations compared to 20 and 50 μM. The most effectiveness of cell death enhancement was at 10 μM of PD98059.

It was found that there was production of hydrogen peroxide and peroxide radicals when DCFH-DA changed to DCF and measured by flow cytometer as shown in Fig. 8. The highest amount of ROS was found at 10 μM and then it decreased due to the fragmentation of nuclei and cytoplasm to form apoptotic bodies as evidenced by agarose gel electrophoresis pattern (Fig. 3) and the morphology of the cells when stained with propidium iodide (data not shown). Another reason is that the apoptotic cells underwent secondary necrosis, which made fluorescence be lower in intensity (not accumulated in the cells).

Discussion

Curcumin is the major compound of food flavoring turmeric (*Curcuma longa* Linn.), and has been used as a herbal medicine. Curcumin shows a variety of physiological effects, and several studies indicate that curcumin is anticarcinogenic (Conney et al., 1991), and anti-inflammatory (Huang et al., 1991). Curcumin further shows antioxidant properties: curcumin acts as a superoxide raidcal scavenger (Reddy and Lokesh, 1994, Ruby et al., 1995) and as a singlet oxygen quencher (Das and Das, 2002). Contrary to the antioxidant nature of curcuminoids, much evidence for cytotoxic properties of curcumin was reported, and its cytotoxicity is suggested to be due to the production of reactive oxygen species.

Curcumin-mediated apoptosis of HL-60 cells was closely related to the increase in the concentration of reactive oxygen species in cells. Generation of reactive oxygen species may be a key factor of induction of apoptosis by curcumin. The effect of antioxidants on HL-60 cell apoptosis induced by curcumin was nonconclusive and required further study. In this process, there was an increase in oxidative stress as shown by the increase in fluorescence of the dye (DCFH-DA to be DCF). The existence of oxidative stress was confirmed that there was hydrogen peroxide and peroxide radical production. But the effect of vitamin C, Trolox, NAC and GSH could have various effects, not just protection of the cells from apoptosis as expected since they all are antioxidants. So, the mechanism of curcumin in inducing apoptosis was complicated. Concerning the combined effect of antioxidants on HL-60 cell apoptosis induced by curcumin, it indicated that Trolox acted as prooxidant rather than antioxidant when combined with vitamin C ($p<0.05$) or combined with GSH ($p<0.05$) as shown in Fig. 2A and Fig. 2C, respectively. The prooxidant effect was also

observed in the combination of vitamin C and NAC ($p<0.05$) as shown in Fig. 2F. However, the single effect of each antioxidant was not significantly different except GSH compared to the treatment with curcumin alone.

The apoptotic death of HL-60 cells demonstrated the appearance of fragmented DNA as shown by the initial band at the low molecular weight of DNA ladder pattern the same as that of positive control (HL-60 cells treated with camtothecin) as shown in Fig. 3. For the mechanism of apoptosis in the system, we found the decrease in mitochondrial membrane potential as depicted in Fig. 4 and Fig. 5, which was in a dose- and time-response manner.

We studied the effect of LY294002 (PI3K inhibitor) and found that it enhanced the mitochondrial membrane potential reduction which meant that the number of apoptotic cells was increased. The mechanism involved PI3K of apoptosis was as followed. In the presence of trophic factor, binding of trophic factors stimulates PI-3 kinase activity, leading to activation of the downstream kinase Akt, which phosphorylates Bad. Phosphorylated Bad then forms a complex with the 14-3-3 protein. With Bad sequestered in the cytosol, the antiapoptotic Bcl-2/Bak-xl proteins can inhibit the activity of Bax, thereby preventing the release of cytochrome c and activation of the caspase cascade (Lodish et al., 2000). In the presence of LY294002, it would inhibit PI3K and induced caspases leading to apoptosis that enhanced the reduction in mitochondrial membrane potential and produced more apoptotic cells (Fig. 6), thus, PI3K is in the signal transduction cascade of HL-60 cell apoptosis. On the contrary, the effect of MEK in the signaling pathway was considered directly. Since the curcumin-induced apoptosis was not inhibited by MEK inhibitor (PD98059) (Fig. 7), it meant that the apoptosis was not via this mediator but through the PI3K. It was reported that mitogen activated protein kinase (MAPK) and phosphotidylinositol-

3-kinase (PI3K/PKB) pathway were relevant to the induction of apoptosis by curcumin in breast cell lines (Squires et al., 2003). However, in HL-60 cells it was not via MAPK when compared to breast cell lines (even though it was the same stimulant, i.e., curcumin). Thus, it depends on the types of cells as well. This was the first report of the roles of combined antioxidants and PI3K on HL-60 cell apoptosis induced by curcumin.

Compared to the concurrent report (Yoshino et al., 2004) as mentioned above, we found that in the system of curcumin-induced apoptosis in HL-60 cells, the oxidative stress existed. Hydrogen peroxide and peroxide radicals were detected by the change of DCFH-DA (nonfluorescence) to be DCF (fluorescence) when there were free radicals in the system as shown in Fig. 8. At 10 μM of curcumin, the amount of ROS was highest that was consistent with Yoshino's work, but in ours the reduction of ROS production at 20 and 30 μM was found. The reduction of ROS corresponded to the morphology of the cells, which showed apoptotic bodies or fragmented bodies under fluorescence microscope (data not shown) and the appearance of fragmented DNA as shown in Fig. 3. The fragmentation could cause less fluorescence per cells or particles when detected with flow cytometer.

In conclusion, each antioxidant and combined antioxidants had different effect on curcumin-induced HL-60 cell apoptosis. Of note, Trolox acted as a prooxidant in addition with vitamin C and GSH. PI3K was in the signal transduction pathway of HL-60 cell apoptosis induced by curcumin.

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Table 1 Statistical values of each antioxidant by Kruskal Wallis analysis

Antioxidant	p value	Significant
Vitamin C	0.261	No
Trolox	0.060	No
GSH	0.031	Yes
NAC	0.263	No

Table 2 Statistical values and significance of combined antioxidants by two way ANOVA.

Antioxidants	p value	Significant
Vitamin C + Trolox	0.001	Yes
Trolox + NAC	0.932	No
Trolox + GSH	0.001	Yes
GSH + NAC	0.322	No
Vitamin C + GSH	0.201	No
Vitamin C + NAC	0.002	Yes

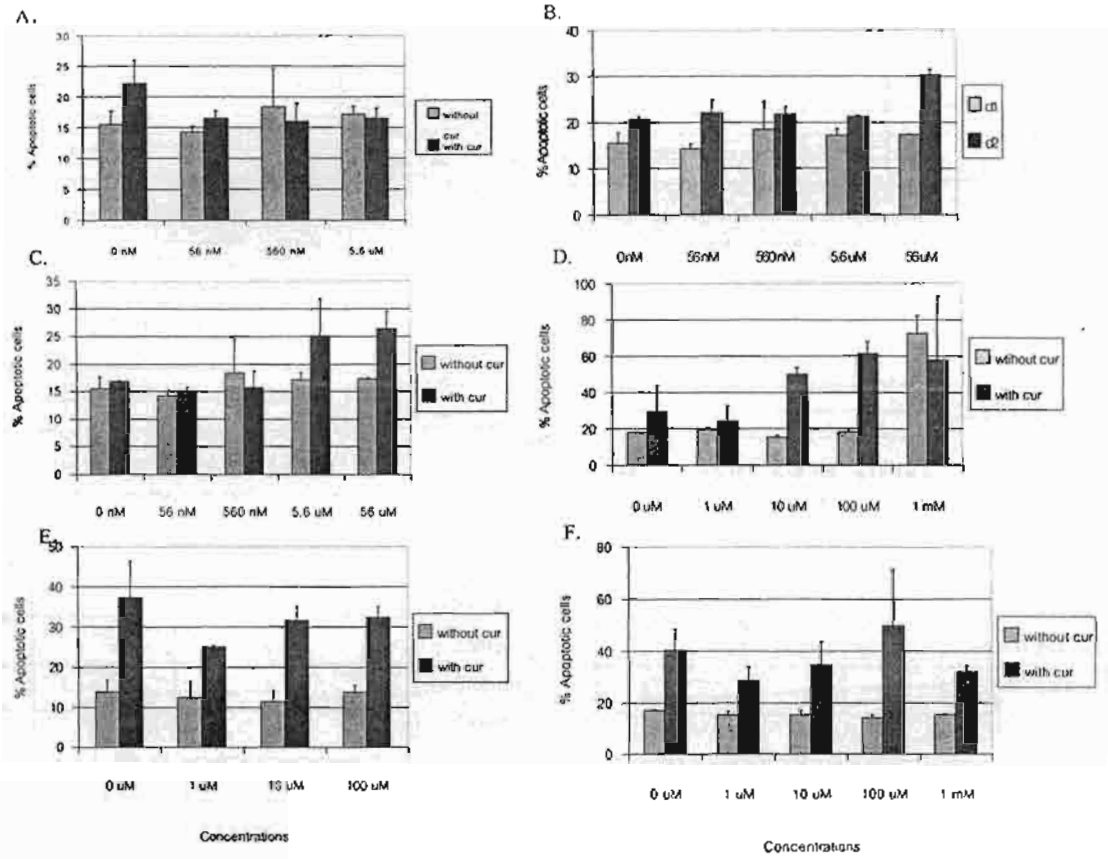

Fig. 1 The effect of antioxidants on HL-60 cell apoptosis. A. HL-60 cells were treated with/without curcumin (10 μM) and with vitamin C at 0, 56, 560 nM and 5.6 μM. B. Cells were treated with vitamin C for 1 and 2 days at various concentrations (0, 56, 560 nM, 5.6 and 56 μM) C. The effect of vitamin C on HL-60 cells when treated with vitamin C for a day and then with curcumin (10 μM) for another day. D. The effect of Trolox on HL-60 cell death at various concentrations (1, 10, 100 μM and 1 mM) and in the status of treatment with curcumin (10 μM). E. The effect of GSH on HL-60 cells when treated with GSH at 1, 10, 100 μM in the status of treatment with/without curcumin (10 μM) ($p<0.05$). F. HL-60 cells were treated with NAC at 0, 1, 10, 100 μM and 1 mM in the status of with/without curcumin (10 μM). When analyzed with Kruskal Wallis analysis, it was significantly different in the group of GSH and curcumin treatment (Fig. 1E: black bars).

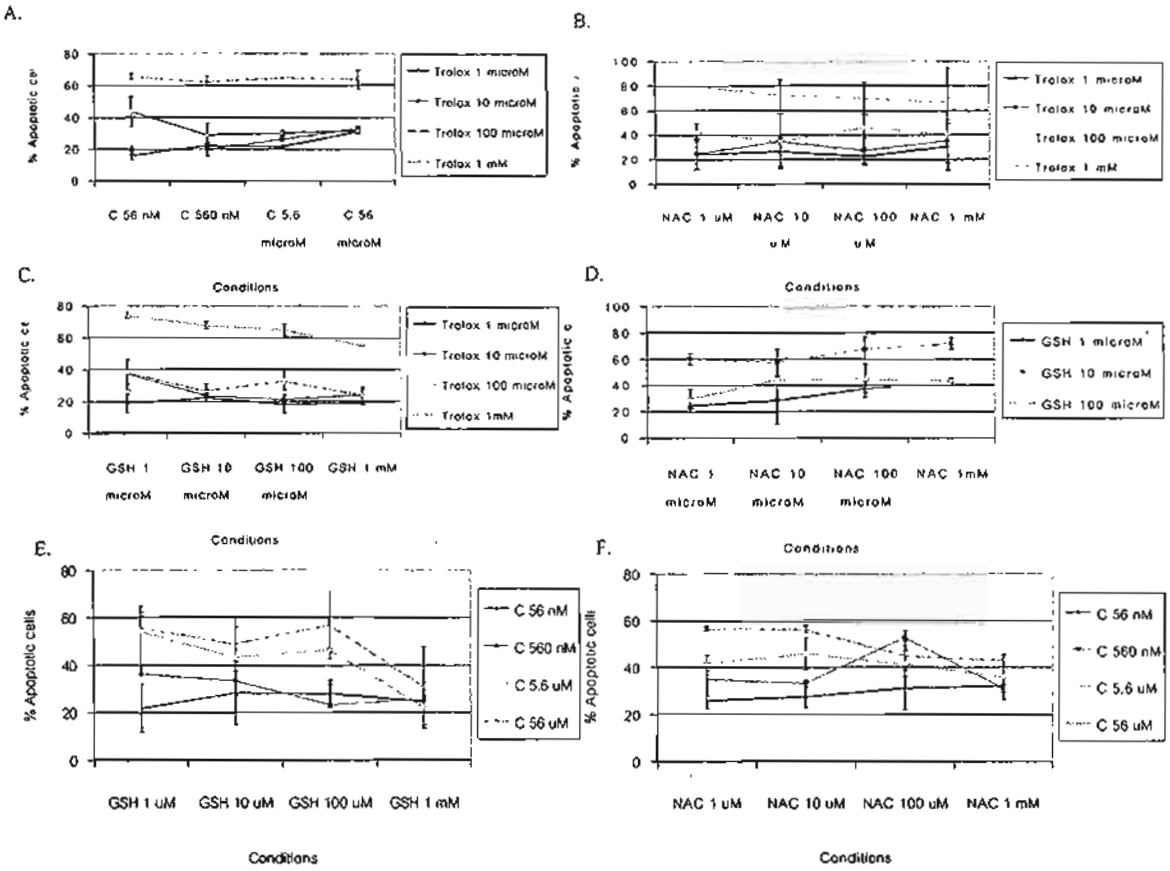

Fig. 2 The combined effect of antioxidants on HL-60 cell apoptosis. A. The effect of vitamin C and Trolox. It was significantly different ($p<0.05$). B. The effect of NAC and Trolox. It was not significant ($p>0.05$). C. The effect of GSH and Trolox. It was significantly different ($p<0.05$). D. The effect of NAC and GSH. It was not significant ($p>0.05$). E. The effect of GSH and vitamin C. It was not significant ($p>0.05$). F. The effect of NAC and vitamin C. It was significantly different ($p<0.05$).

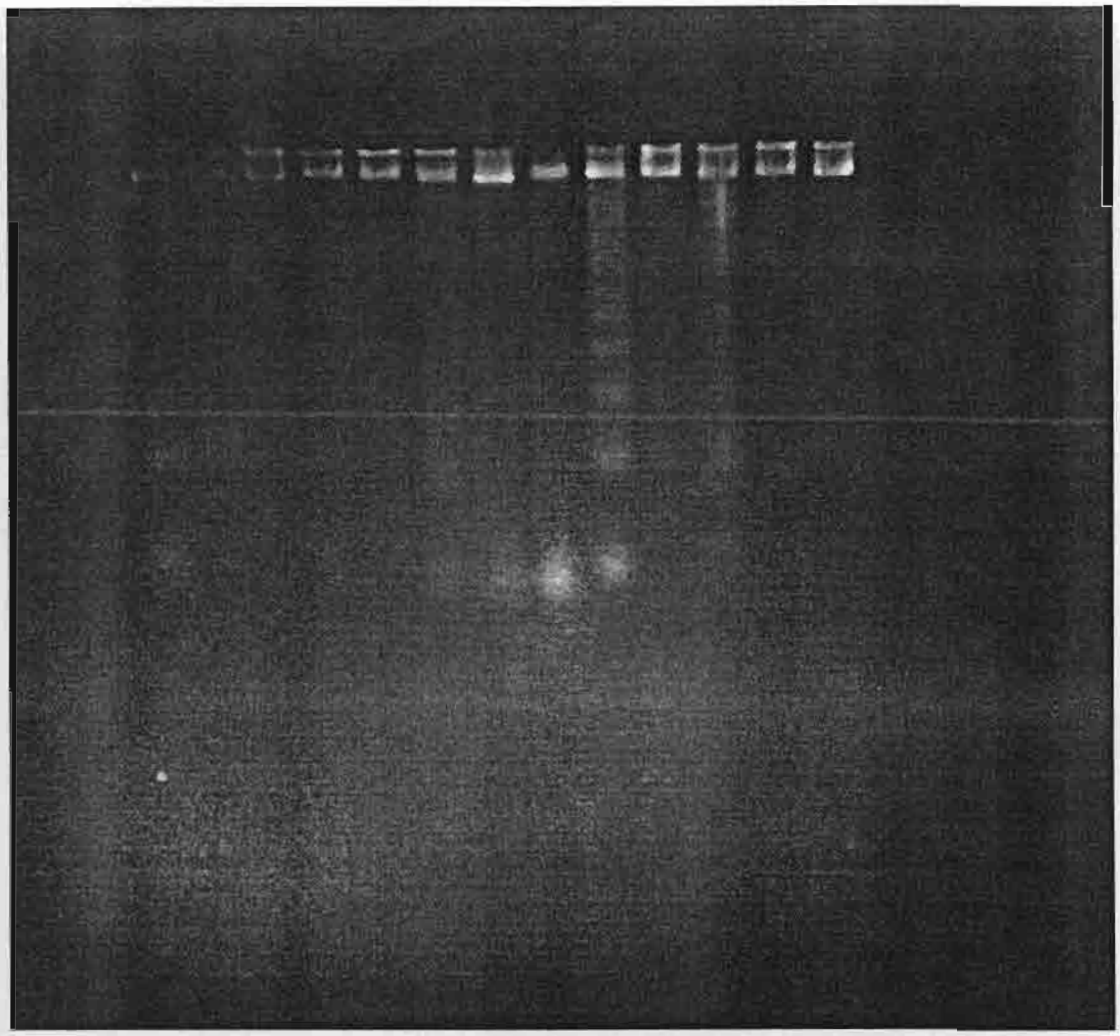

Fig. 3 Agarose gel electrophoresis pattern of HL-60 cells treated with vitamin C and curcumin simultaneously. Lane 1 HL-60 cells treated with camtothecin; lane 2 negative control; lane 3 HL-60 cells treated with vitamin C 56 nM + curcumin 10 μM; lane 4 HL-60 cells treated with vitamin C 0.56 μM + curcumin 10 μM; lane 5 HL-60 cells treated with vitamin C 5.6 μM + curcumin 10 μM. Lane 6 HL-60 cells treated with curcumin 10 μM; lane 7 HL-60 cells treated with curcumin 15 μM; lane 8 HL-60 cells treated with curcumin 30 μM. Lane 9 was HL-60 cells treated with camtothecin. Lane 10 negative control (without treatment); lane 11 HL-60 cells treated with 0.1 mM hydrogen peroxide; lane 12 HL-60 cells treated with 1 mM hydrogen peroxide; lane 13 HL-60 cells treated with10 mM hydrogen peroxide. There was ladder pattern in lane 1 and 9, which were positive control. Lane 6-8 (HL-60 cells treated with curcumin 10, 15 and 30 μM, respectively) showed small DNA fragment which was the initial band at low molecular weight of the ladder pattern. Lane 11 also demonstrated of ladder pattern (0.1 M hydrogen peroxide treated cells).

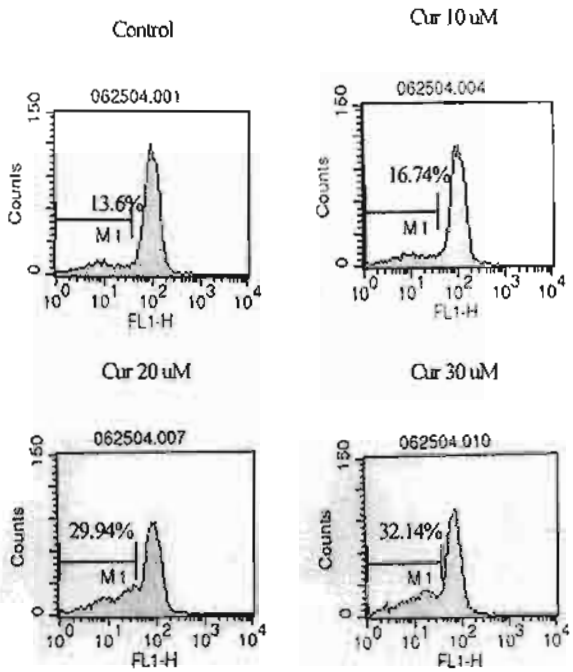

Fig. 4 Reduction of mitochondrial membrane potential when HL-60 cells were treated with curcumin at 0, 10, 20 and 30 μM for 4 h. Aliquots of $1x10^6$ cells were incubated with 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$, 50 nM] and analyzed by flow cytometry. The percentages reflected the reduction of mitochondrial membrane potential.

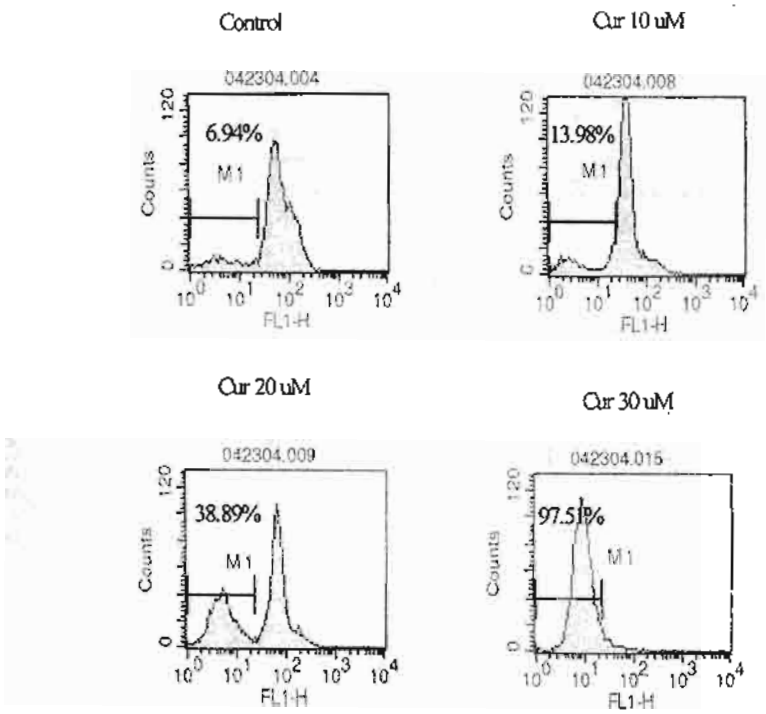

Fig. 5 Reduction of mitochondrial membrane potential when HL-60 cells were treated with curcumin at 0, 10, 20 and 30 μM for 24 h.

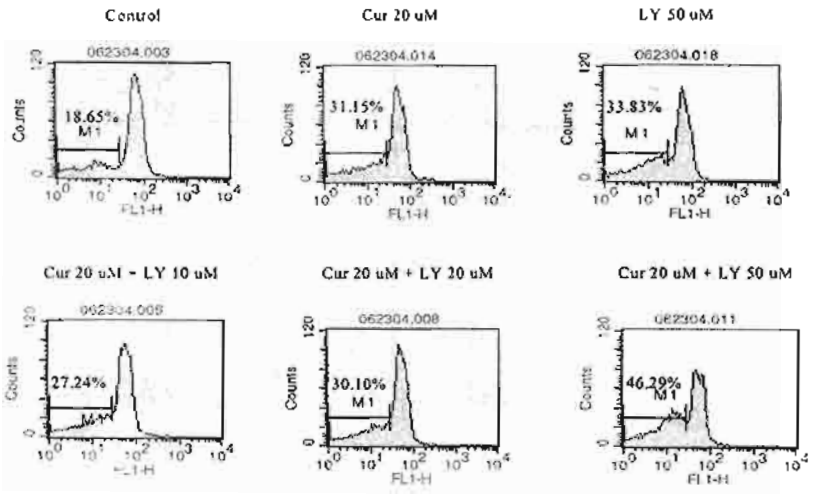

Fig. 6 The pattern of mitochondrial membrane potential when HL-60 cells were pretreated with LY294002 (PI3K inhibitor) at 10, 20 and 50 μM for 50 min and then with curcumin at 20 μM for 4 h or with curcumin 20 μM alone for 4 h or with LY294002 50 μM alone for 4 h. Then $DiOC_6(3)$ was added at 40 nM for 15 min at 37 °C and measured for mitochondrial membrane potential by using flow cytometry as mentioned in Materials and Methods.

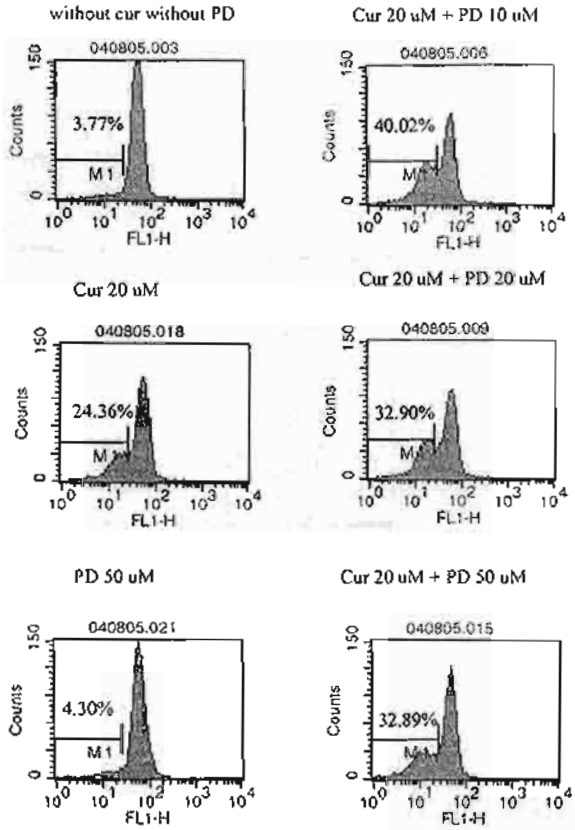

Fig. 7 The effect of PD98059 (MEK or MAP kinase kinase inhibitor) on HL-60 cell apoptosis induced by curcumin. HL-60 cells were pretreated with PD98059 for 50 min and then incubated with curcumin (20 μM) for 4 h. The HL-60 cells treated with curcumin (20 μM) alone or PD98059 alone (50 μM) were compared. The mitochondrial membrane potential was measured as mentioned in Materials and Methods.

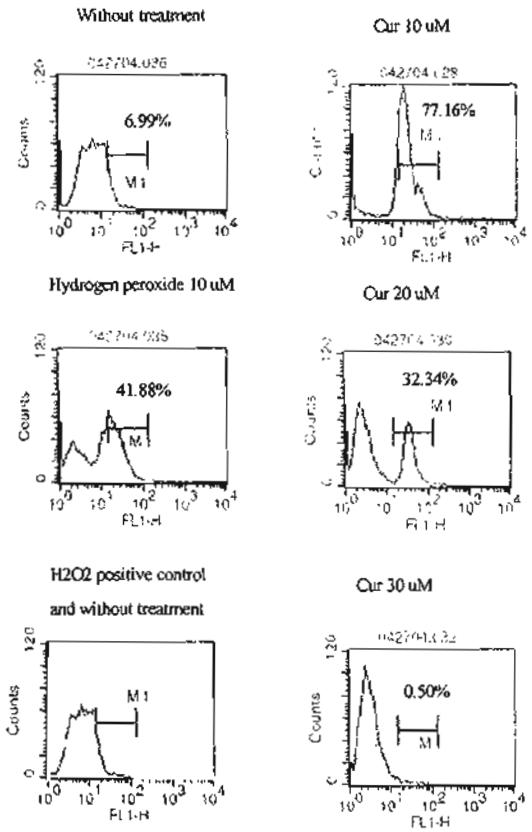

Fig. 8 The production of ROS (peroxides and hydrogen peroxides detected by the change of DCFH to DCF) after curcumin treatment for 4 h. The open peak at left lower picture was a positive control after 10 mM hydrogen peroxide treatment (overlay).

CURCUMIN INDUCED APOPTOSIS IN HL-60 CELLS: THE EFFECT OF ANTIOXIDANTS

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Abstract

Curcumin is the main biologically active phytochemical compound in turmeric. Curcumin has anti-inflammatory, antioxidant, anticarcinogenic and antiproliferative activities. The research aims were to identify the mode and mechanism of cell death of human promyelocytic leukemic (HL-60) cells induced by curcumin and to determine the effects of vitamin C, Trolox, reduced glutathione (GSH) and *N*-acetylcysteine (NAC) on this process. HL-60 cells were incubated with curcumin for 24 h and apoptotic cells were quantitated by flow cytometry following staining with annexin V-FITC and propidium iodide. Curcumin induced apoptosis in a dose-dependent manner, which was also confirmed by the decrease in mitochondrial membrane potential. PI3K inhibitor (LY294002) and MEK inhibitor (PD98059) had the effect on the apoptotic cell death induced by curcumin (20 μM). There was an increase in free radical generation, as measured by dichlorofluorescein diacetate and flow cytometry, indicating the existence of oxidative stress in curcumin-treated HL-60 cells. In the presence of 10 μM curcumin, vitamin C (56 nM, 560 nM and 5.6 μM), and GSH (10 μM, 100 μM and 1 mM) reduced the number of apoptotic cells, but NAC and Trolox had a dual effect, being protective at 1 μM, 10 μM and 1 mM for NAC and 1 μM for Trolox; and synergistic at 100 μM for NAC and 10, 100 μM and 1 mM for Trolox.

Keywords: apoptosis, curcumin, HL-60 cells, PI3K inhibitor, MEK inhibitor, antioxidants

Abbreviations: HL-60 cells, human promyelocytic leukemic cells; GSH, glutathione; NAC, N-acetylcysteine; PI3K, phosphotidylinositol 3-kinase; MAPKK, mitogen activated protein kinase kinase

Introduction

Apoptosis is a physiological suicide mechanism that preserves homeostasis, in which cell death naturally occurs during tissue turnover (1). It is the most common form of eukaryotic cell death. Cells undergoing apoptosis display profound structural changes, including plasma membrane blebbing and nuclear disintegration. The nuclear change is associated with extensive damage to chromatin and DNA cleavage into nucleosomal-length DNA fragments after activation of calcium-dependent endogenous endonucleases (2). Apoptosis is essential in many physiological processes, including the embryonic development and the maturation of immune system (3). It is currently the subject of intense research, partially because tumor cells are susceptible to death by apoptosis in response to drugs and/or radiation treatment.

Curcumin, also known as diferuloylmethane (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), is the major yellow pigment extracted from turmeric, which is used extensively in curries. Its properties as a coloring and flavoring agent have led to uses as a dietary additive in variety of foods (4, 5). Extracts containing curcumin have also been used in medicines in India and Southeast Asia for generations, and according to tradition are useful in the treatment of inflammation, skin wounds, hepatic and biliary disorders, cough, as well as certain tumors (6).

Since there was a report that curcumin-mediated apoptosis was related to the increase in intracellular reactive oxygen species (7). We aimed to demonstrated the effect of four antioxidant molecules, i.e. ascorbic acid, glutathione (GSH), N-acetylcysteine (NAC) and Trolox (a water soluble vitamin E), on HL-60 cell apoptosis. Moreover, since many researchers studied of the mechanisms of curcumin-induced apoptosis in HL-60 cells and reported of the significance of mitochondrial leakage of cytochrome. It was intriguing to study whether the mitochondrial membrane potential changed and the role of PI3K and MEK/MAPKK in the apoptotic cell death in HL-60 cells induced by curcumin.

Materials and Methods

RPMI-1640 and fetal bovine serum were obtained from Gibco-BRL. Annexin V-FITC kit was obtained from Roche. Curcumin (1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione), glutathione, N-acetylcysteine, vitamin C and 3,3'-dihexyloxacarbocynine iodide or $DiO_6(3)$ were obtained from Sigma. Trolox was obtained from Aldrich. LY294002 and PD98059 were obtained from Calbiochem.

Cell culture and treatment

HL60 cells were cultured in 10% fetal bovine serum in RPMI-1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 μg/ml) and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The preconfluent (growth phase) cells were treated with curcumin (at concentrations as indicated) for 24 hours. Diluted curcumin solutions were prepared in alcohol (0.5% final concentration). It was found that 0.5% alcohol did not affect cell viability. After incubation the cells with curcumin for 24 hours, the cells were examined under fluorescence microscope and processed through flow cytometry.

In the conditions of treatment with the antioxidant, vitamin C or Trolox or N-acetylcysteine or glutathione, it was added simultaneously with curcumin (10 μM). The concentrations of vitamin C were varied from 56 nM, 560 nM, 5.6 μM, and 56 μM. For Trolox, the concentrations were varied from 1 μM, 10 μM, 100 μM, and 1 mM. For Nacetyl-cysteine, the concentrations were 1, 10, 100 μM and 1 mM. For glutathione, the doses were 1, 10, and 100 μM. Then the cells were processed with flow cytometer.

HL-60 cells were pretreated with LY294002 or PD98059 at 10, 20 and 50 μM for 50 minutes and then with curcumin at 20 μM for 4 hours or with curcumin 20 μM alone or with LY294002 or PD98059 at 50 μM alone for 4 hours. Then $DiOC_6(3)$ were added at 40 nM for 15 minutes at 37 °C and measured for mitochondrial membrane potential.

Flow cytometry (8)

HL60 cells were collected at the concentration of 10^6 cells. The cells were washed once and then centrifuged at 200 x g to get the cell pellets which were resuspended in 100 μl of the binding buffer provided by the reagent kit. Annexin V-FITC (2 μl) and propidium

iodide (2 μl) were added in each tube. Then they were incubated at room temperature for 15 min in the dark. Finally the binding buffer, 900 μl, were added and mixed for further processing.

Mitochondrial membrane potential measurement (9)

For mitochondrial membrane potential ($\Delta\Psi$) and intracellular ROS measurement, $5x10^5$ cells were incubated for 15 minutes at 37 °C with 3,3'-dihexyloxacarbocynine iodide [DiO6(3), 40 nM] followed by FACScan (Becton Dickinson) analysis.

Statistical Analysis

The tests were performed in duplicate independently in 3 experiments and analyzed based on Kruskal Wallis analysis (one way ANOVA). For the two variables the data were analyzed by two way ANOVA.

Results

When the cells were treated with vitamin C simultaneously with curcumin, it reduced the apoptotic cells at dose 56, 560 nM and 5.6 μM. Thus, vitamin C had protective effect on apoptotic cell death as shown in Fig. 1A. It was statistically non-significant ($p>0.05$). But when the cells were treated with vitamin C for 1 and 2 days, it was demonstrated that the percentages of apoptotic cells were increased at all concentrations of vitamin C (56, 560 nM, 5.6 and 56 μM). This meant that vitamin C was not able to inhibit the apoptosis on day 2 (Fig. 1B). The number of apoptotic cells on day 1 and day 2 was not different significantly ($p>0.05$).

Then the HL-60 cells were treated with vitamin C first for 1 day and then with curcumin for another day, it was found that the percentage of apoptotic cells was increased when compared to treatment with vitamin C alone (Fig. 1C). It meant that the treatment of vitamin C first for a day could not prevent cells from apoptosis but in contrast it increased the number of apoptotic cells. Hence, the pretreatment of vitamin C had a synergistic effect on apoptosis when induced with curcumin except at the concentrations of vitamin C at 56 and 560 nM which could reduce the number of cell death compared to treatment with curcumin alone. However, it was not significantly different ($p>0.05$).

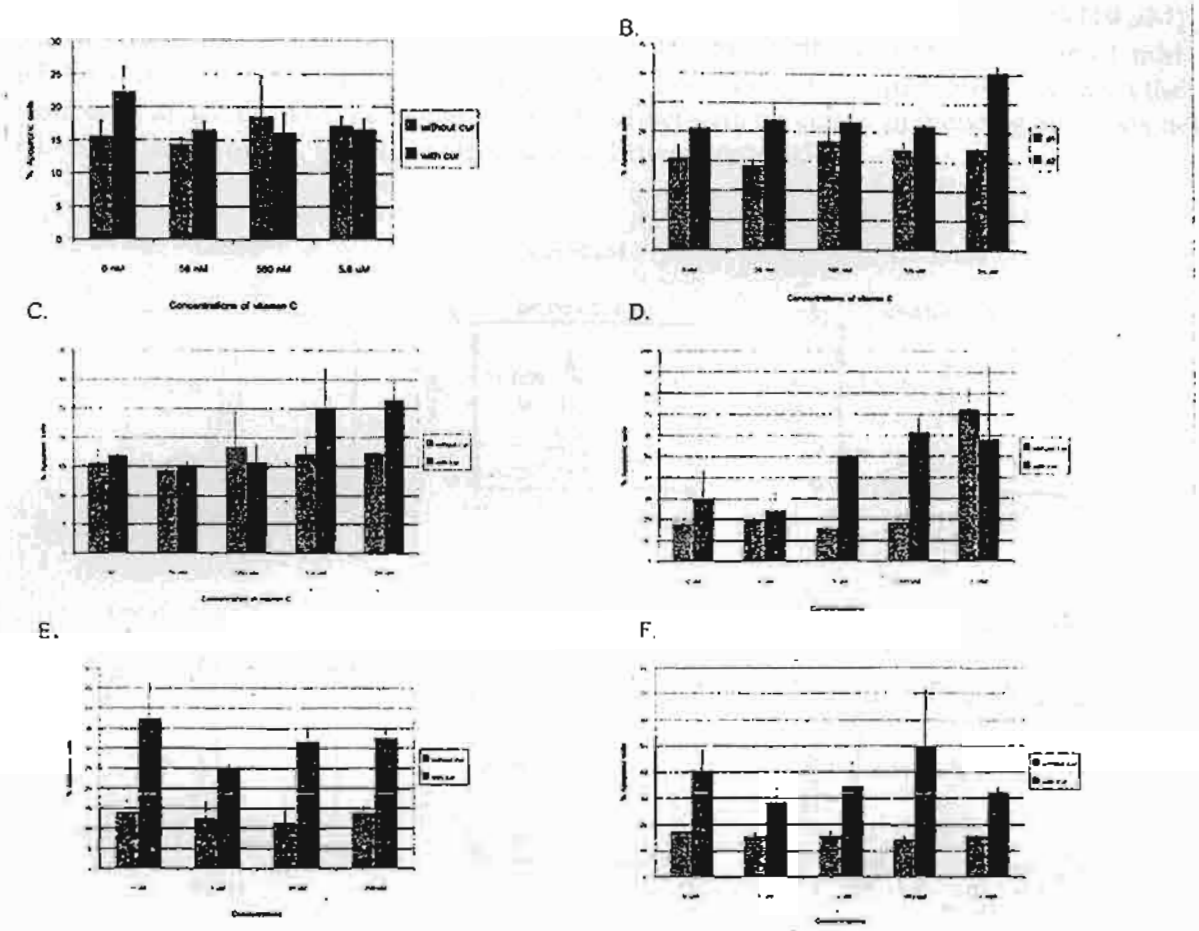

Fig. 1 The effect of antioxidants on HL-60 cell apoptosis. A. HL-60 cells were treated with/without curcumin (10 μM) and with vitamin C at 0, 56, 560 nM, and 5.6 μM. B. Cells were treated with vitamin C for 1 and 2 days at concentrations of 0, 56, 560 nM, 5.6 and 56 μM. C. The effect of vitamin C on HL-60 cells when treated with vitamin C for a day and then with curcumin (10 μM) for another day. D. The effect of Trolox on HL-60 cell death at various concentrations (1, 10, 100 μM and 1 mM) and in the status of treatment with/without curcumin (10 μM). E. The effect of GSH on HL-60 cells when treated with GSH at 1, 10, 100 μM and with/without curcumin (10 μM). F. HL-60 cells were treated with NAC at 0, 1, 10, 100 μM and 1 mM in the status of with/without curcumin (10 μM).

It was found that when the HL-60 cells were treated with Trolox alone, it did not cause cells to die more at doses of 1, 10, and 100 μM but at concentration of 1 mM it caused the cells to undergo apoptosis 4 folds compared to control. However, in the status of treatment with curcumin 10 μM, it was found that Trolox cause the cells to undergo apoptosis more than control (curcumin 10 μM alone) at 10 μM, 100 μM, and 1 mM; whereas at the concentration of 1 μM it reduced the number of apoptotic cells. This meant that Trolox had dual effect. It was a prooxidant at 10 and 100 μM and 1 mM and showed the inhibitory effect of HL-60 cell apoptosis at 1 μM as shown in Fig. 1D. However, it was not significantly different ($p>0.05$).

Glutathione (GSH), an antioxidant found mainly in the red blood cells and also in other mammal cells, was also tested. It was found that GSH (at 1, 10, and 100 μM) could reduce the number of apoptotic cells compared to the system when treated with curcumin alone as shown in Fig. 1E. It was statistically significant ($p<0.05$).

The effect of N-acetylcysteine on HL-60 cell apoptosis induced by curcumin (10 μM) was shown in Fig. 1F. NAC had dual effect, i.e. at concentrations of 1, 10 μM and 1 mM inhibited the HL-60 cell apoptosis. Whereas at concentration of 100 μM, NAC increased the number of apoptotic cells, i.e. it had synergistic effect with curcumin in inducing apoptosis in HL-60 cells. However, it was not significantly different ($p>0.05$).

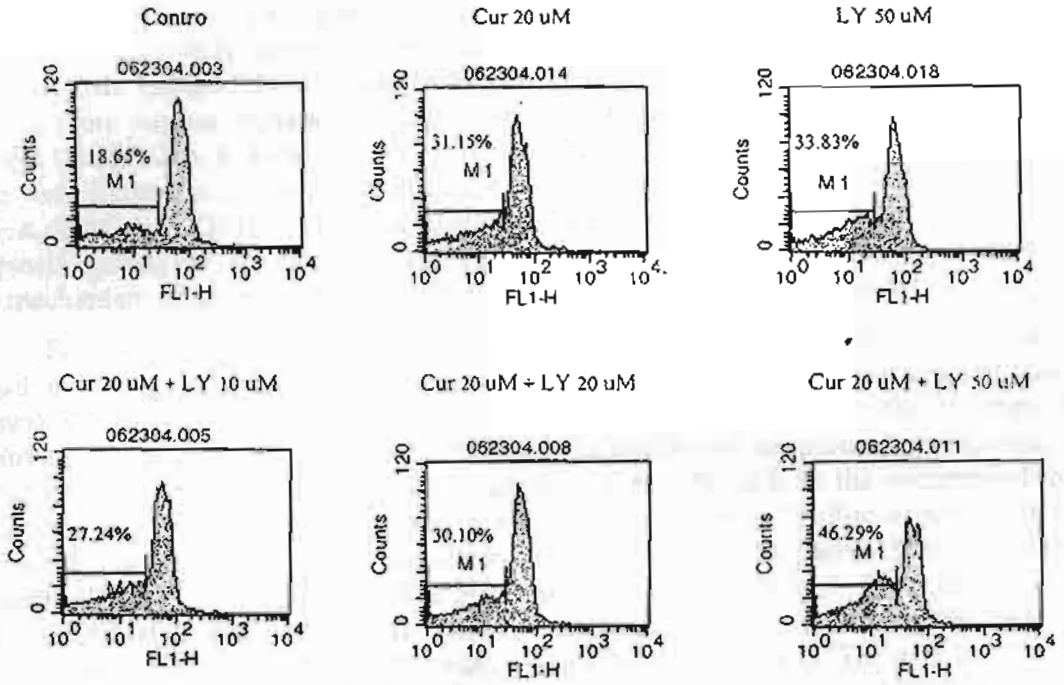

Fig. 2 The pattern of mitochondrial membrane potential when HL-60 cells were pretreated with LY294002 (a PI3K inhibitor) at 10, 20 and 50 μM for 50 minutes and then with curcumin at 20 μM for 4 hours or with curcumin 20 μM alone or with LY294002 50 μM alone for 4 hours. Then $DiOC_6(3)$ were added at 40 nM for 15 minutes at 37 °C and measured mitochondrial membrane potential by using flow cytometry as mentioned in Materials and Methods.

When HL-60 cells were treated with 50 μM of LY294002 for 50 min then with curcumin (20 μM) for another 4 h. The reduction in mitochondrial membrane potential was up to 46.29% which meant that it increased the HL-60 cell apoptosis compared to that treated with curcumin (20 μM) alone (31.15%) as shown in Fig. 2. But when the HL-60 cells were treated with PD98059 it caused more in the reduction of mitochondrial membrane potential when compared to the system that treated with curcumin (20 μM) alone (data not shown). When treating the cells with PD98059 (10 μM) the mitochondrial membrane potential reduction was 40.02%, at 20 μM of PD98059 it was 32.90%, and at PD98059 50 μM its reduction was 32.89%. It seemed that at low concentration of PD98059 it could cause or enhance the reduction more than at higher concentrations. The most effectiveness of cell death enhancement was at 10 μM of PD98059.

Discussion

Curcumin is the major compound of food flavoring turmeric (Curcuma longa Linn.), and has been used as a herbal medicine. Curcumin shows a variety of physiological effects,

and several studies indicate that curcumin to be anticarcinogenic (10), and anti-inflammatory (11). Curcumin further shows antioxidant properties: curcumin acts as a superoxide radical scavenger (12, 13) and as a singlet oxygen quencher (14). Contrary to the antioxidant nature of curcuminoids, much evidence for cytotoxic properties of curcumin was reported, and its cytotoxicity is suggested to be due to production of reactive oxygen species.

Curcumin-mediated apoptosis of HL-60 cells was closely related to the increase in the concentration of reactive oxygen species in cells. Generation of reactive oxygen species may be a key factor of induction of apoptosis by curcumin. The effect of antioxidants on HL-60 cell apoptosis induced by curcumin showed many aspects of unclearness. Since in this process, there was an increase in oxidative stress as shown by the increase in fluorescence of the dye (DCFH-DA to be DCF) (7). The existence of oxidative stress was confirmed that there were hydrogen peroxide and peroxide radical production. But the effect of vitamin C, Trolox, NAC and GSH could have various effects, not just protection of the cells from apoptosis as they all are antioxidants. But some of them also contained prooxidant effect. The mechanism of action of curcumin in inducing apoptosis was complicated.

For the mechanism of apoptosis in HL-60 cells, we found the decrease in mitochondrial membrane potential that was in a dose- and time-response manner (data not shown). We studied the effect of LY294002 (a PI3K inhibitor) and found that it enhanced the membrane potential reduction, which meant that the number of apoptotic cells was increased. The mechanism involved the PI3K of apoptosis was as followed. In the presence of trophic factor, binding of trophic factors stimulates PI-3 kinase activity, leading to activation of the downstream kinase Akt, which phosphorylates Bad. Phosphorylated Bad then forms a complex with the 14-3-3 protein. With Bad sequestered in the cytosol, the antiapoptotic Bcl-2/Bak-xl proteins can inhibit the activity of Bax, thereby preventing the release of cytochrome c and activation of the caspase cascade (15). Thus, PI3K is in the inhibitory cascade of apoptosis. In the presence of LY294002 it would inhibit PI3K and induced apoptosis that caused more apoptotic cells (Fig. 2). At the same time, the effect of MAPKK/MEK in the signal transduction pathway was examined. Since the curcumin-induced apoptosis were not inhibited by PD98059, it meant that the apoptosis was not via this mediator but through the PI3K. It was reported that mitogen activated protein kinase (MAPK) and phosphotidylinositol-3-kinase (PI3K/PKB) pathway were relevant to the induction of apoptosis by curcumin in breast cell lines (16). This was the first report of the roles of four antioxidants and PI3K on HL-60 cell apoptosis induced by curcumin. Should curcumin be used in the clinical aspects requires further in vivo experiments.

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CURCUMIN-INDUCED HL60 CELL NECROSIS *IN VITRO*

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Background Curcumin is the main biologically active phytochemical compound in turmeric. Curcuminoid (ethanolic extract) was found to contain curcumin: demethoxycurcumin: bisdemethoxycurcumin equaled to 48:21:31. The curcuminoid (isopropanolic extract) was found to have the three compounds at the ratio of 86:13:1. Curcumin (commercial grade, e.g. Sigma) contained the three components in the ratio of 77:20:3, respectively. Curcumin has anti-inflammatory, antioxidant, anticarcinogenic and antiproliferative activities.

Objective To identify the mode and amount of cell death induced by curcuminoids and curcumin on human promyelocytic leukemic (HL60) cells.

Methods HL60 cells were incubated with curcumin (Sigma) or curcuminoids (ethanolic or isopropanolic extracts) at 1, 10, 100 ng/mL and 1, 10 μg/mL and the mode of cell death was determined by using annexin V-FITC and propidium iodide staining and flow cytometry. The cells were incubated with curcumin or curcuminoids (ethanolic or isopropanolic extracts) for 1 or 2 days and the percentages of cell death were compared.

Results Curcumin (Sigma) induced HL60 to undergo necrotic cell death (95.1%) at 10 μg/mL. At the concentration of 1 ng/mL-1 μg/mL, the number of cell death was not significantly different from control (without treatment). When the incubation time was increased to 2 days, the cells which died via necrosis reached 96% at 10 μg/mL. Therefore, the incubation time was chosen to be 1 day through out the study. HL60 cells underwent greater level of necrosis at high concentrations (and 10 μg/mL) of the three compounds than at lower concentrations (1, 10, 100 ng/mL) ($p < 0.05$). It was found that curcumin (Sigma) exhibited the dose response relationship of inducing necrosis at the concentration of 1, 2, 4 and 8 μg/mL. The percentages of necrotic cell death due to the ethanolic extract, isopropanolic extract and curcumin (Sigma) at the concentration of 10 μg/mL were 25, 98 and 95%, respectively. The most potent preparation was isopropanolic extract whereas the least effective was cucuminoid (ethanolic extract).

Conclusion Curcumin could induce HL60 cells to undergo necrosis. Additional studies are needed to determine whether curcumin and other curcuminoids will be useful as a complementary treatment for human leukemia.

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The Effect of Antioxidants on HL60 Cell Death Induced by Curcumin (P-I)

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Objective : To determine the effects of vitamin C, Trolox (vitamin E), glutathione (GSH) and *N*-acetylcysteine (NAC) on death of human promyelocytic leukemic (HL60) cells induced by curcumin.

Methods : HL60 cells were treated with vitamin C, Trolox, GSH, and NAC at various concentrations for 24 hours (each alone) or simultaneously with curcumin. The numbers of dead cells and type of cell death were determined by flow cytometry and staining with Annexin V-FITC and propidium iodide.

Results : Curcumin could induce necrotic HL60 cell death in a dose dependent manner with maximal effect at 8 μg/ml which caused 95±5.4% ($p<0.05$) cells to die. The dose of 4 μg/ml was chosen for further experiments as this caused cell death at 35.6±10.5%. Vitamin C caused a mild degree of necrosis (16.2±3.6%) at 0.56 _M. GSH and NAC had no cytotoxic effect on HL60 cells at all the concentrations used. Trolox could induce 21.3±3.7% and 82.4±13.5% HL60 necrosis at concentration of 100 μM and 1 mM, respectively ($p<0.05$). Vitamin C reduced the percentages of necrotic cell death when treated in combination with curcumin ($p>0.05$). When added with curcumin Trolox increased the amount of necrotic cell death ($p<0.05$). When treated with curcumin, GSH could inhibit necrotic cell death ($p<0.05$) but not in a dose dependent manner. However, NAC had a biphasic effect on curcumin-induced cell death, namely, NAC at 100 _M was synergistic, but became inhibitory at lower and higher doses.

Conclusion : Vitamin C and GSH had inhibitory effects on curcumin-induced necrosis of HL60 cells. On the other hand, Trolox had a pro-oxidant effect that synergistically enhanced HL60 cell death, whereas NAC showed a biphasic effect depending on the dose used. These phenomena should be taken into consideration should these compounds be used in the treatment of leukemic diseases.

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