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

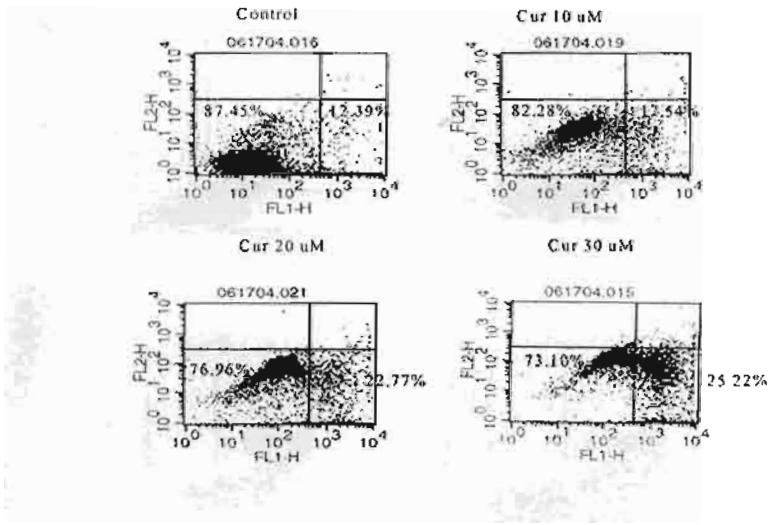


Fig. 1 The effect of curcumin at various concentrations (10, 20, and 30 μM) on HL-60 cell apoptosis incubated for 4 h. This is the representative of three experiments which were performed.

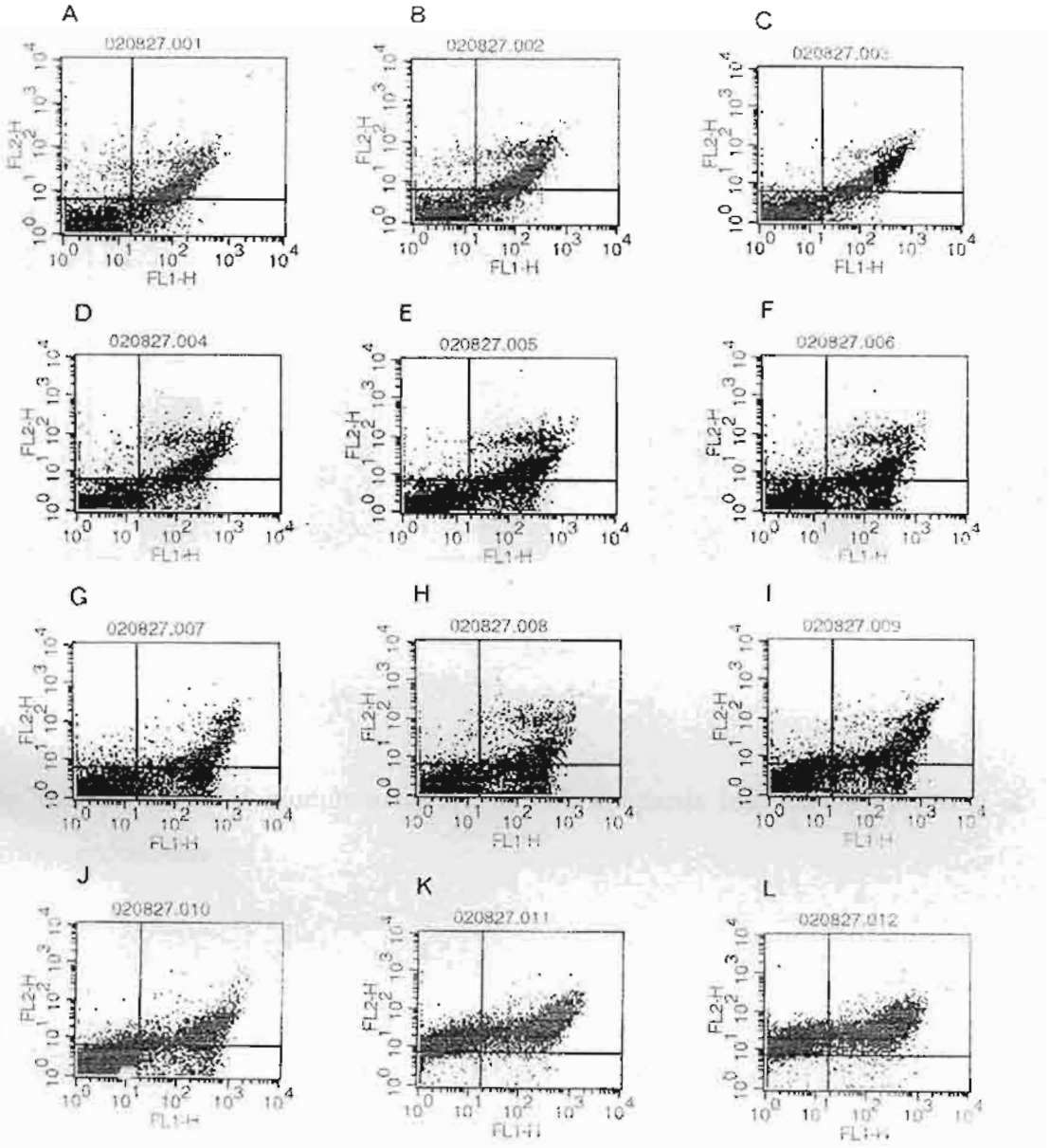


Fig. 2 Dot plots of the effect of curcuminoid (isopropanol extract) on HL-60 cell apoptosis at various concentrations for 24 h. A, B control or without treatment; C, D cells treated with curcuminoid (1 ng/ml); E, F cells treated with curcuminoid (10 ng/ml); G, H cells treated with curcuminoid (100 ng/ml);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

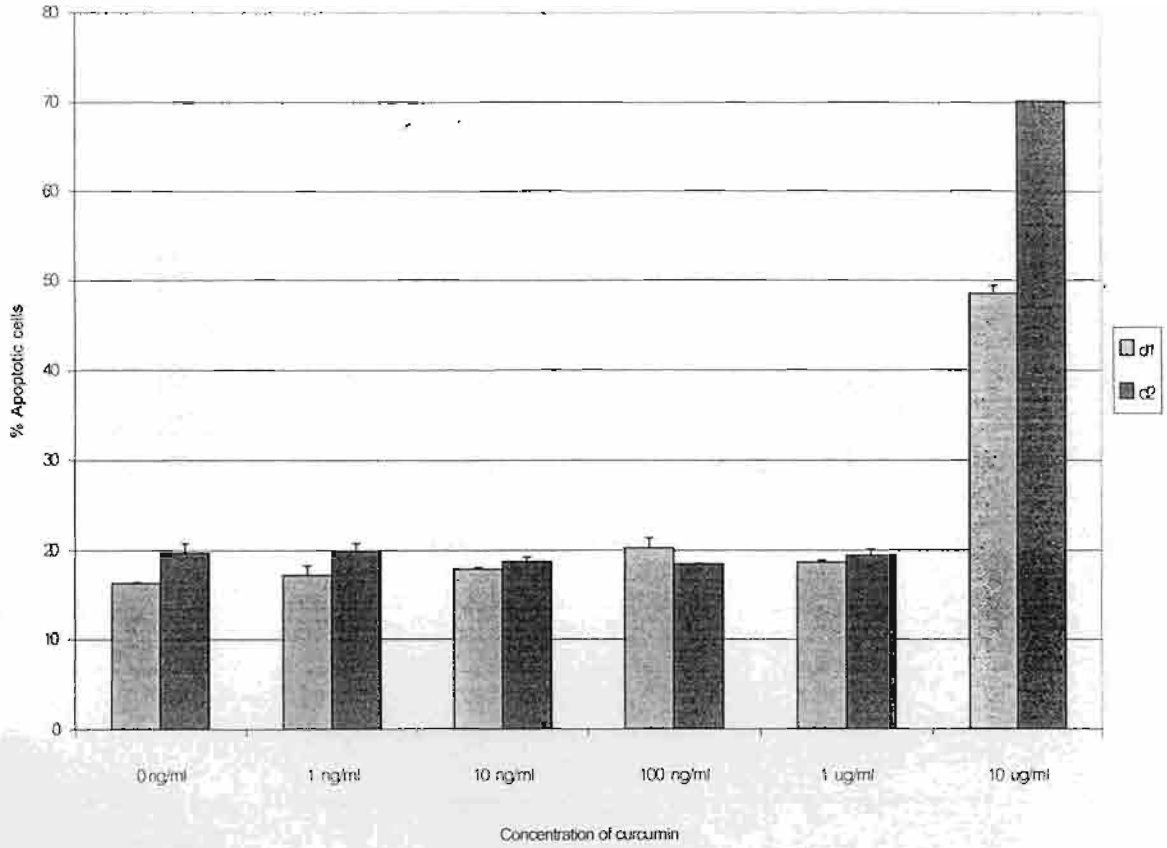
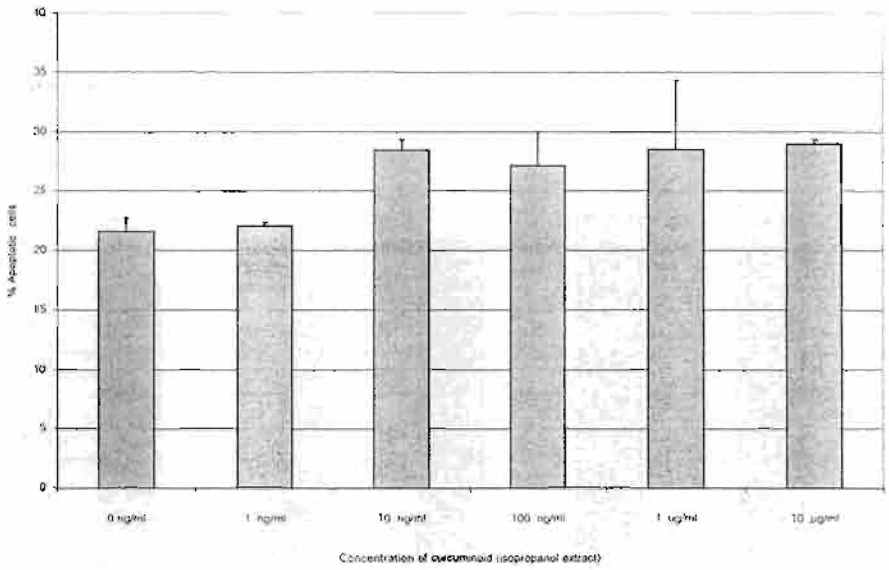


Fig. 4 The effect of curcumin on HL-60 cell apoptosis at various concentrations and at incubation time of 1 and 2 days.

A.



B.

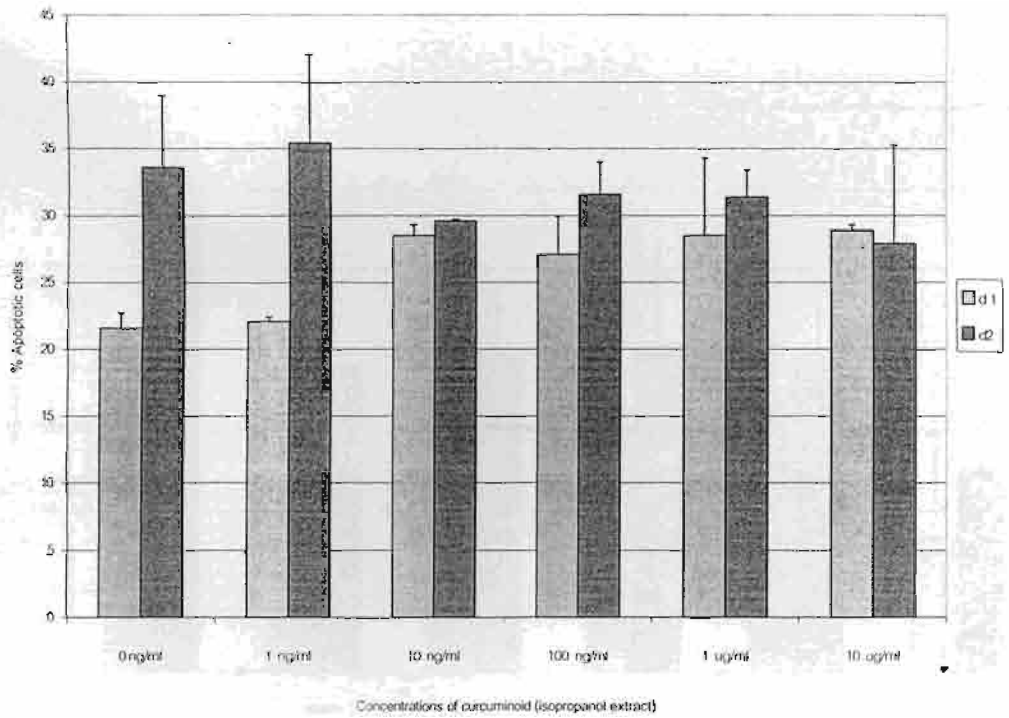
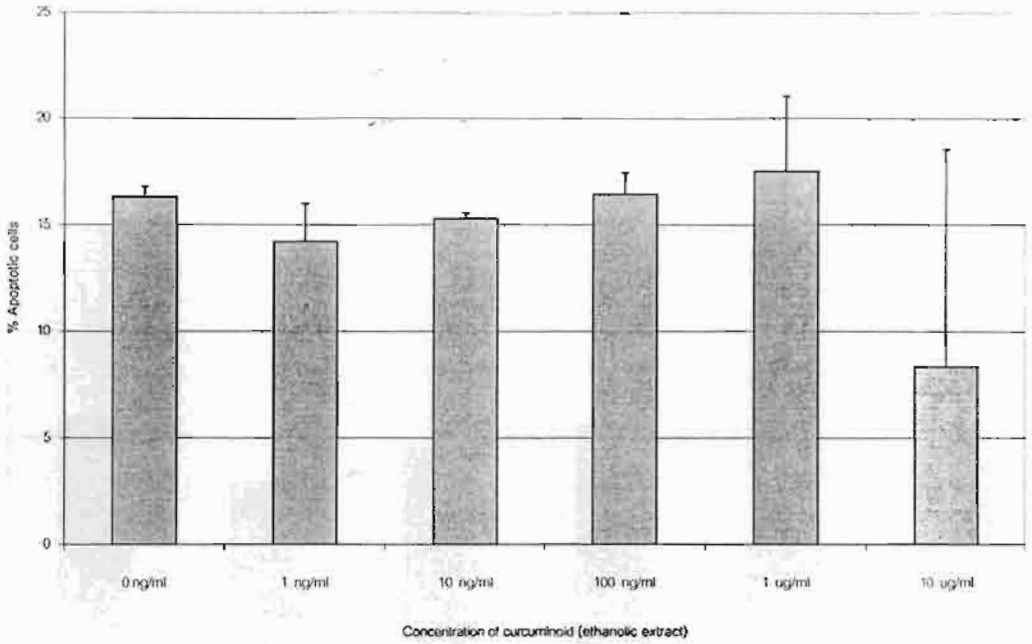


Fig. 5 The effect of curcuminoid extracts (isopropanol extract) on HL-60 cell apoptosis at various concentrations on day 1 (A) and comparison between day 1 and day 2 was shown in (B).

A.



B

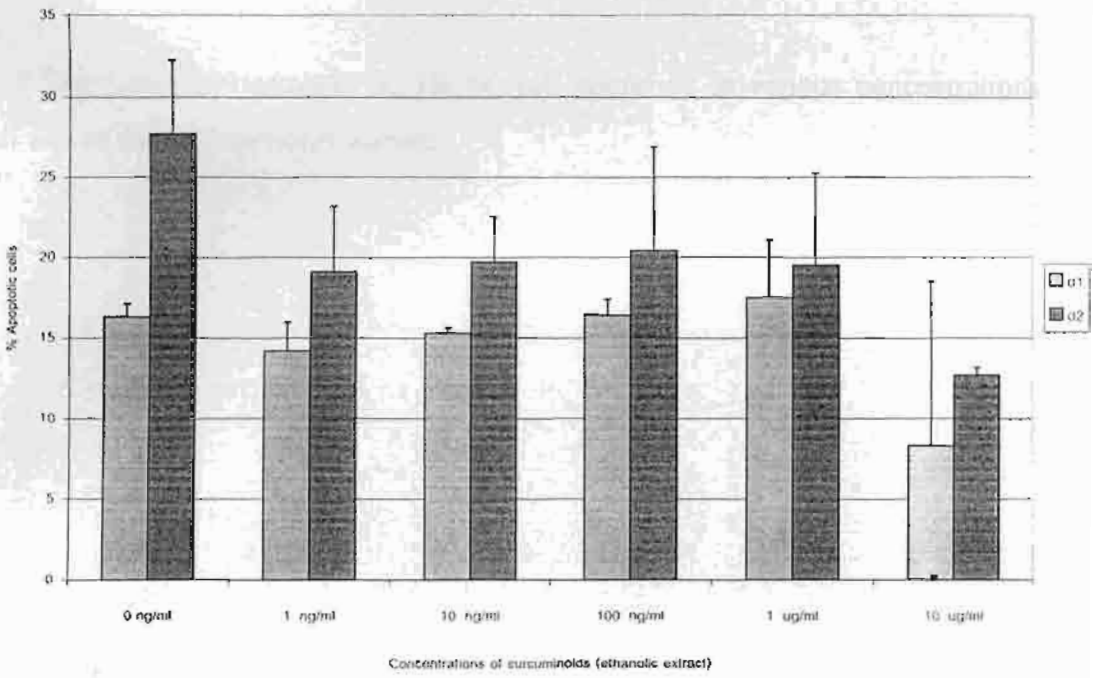


Fig. 6 The effect of curcuminoid extract (ethanolic extract) on HL-60 cell apoptosis at various concentrations on day 1 (A) and comparison between day 1 and day 2 was shown in (B).

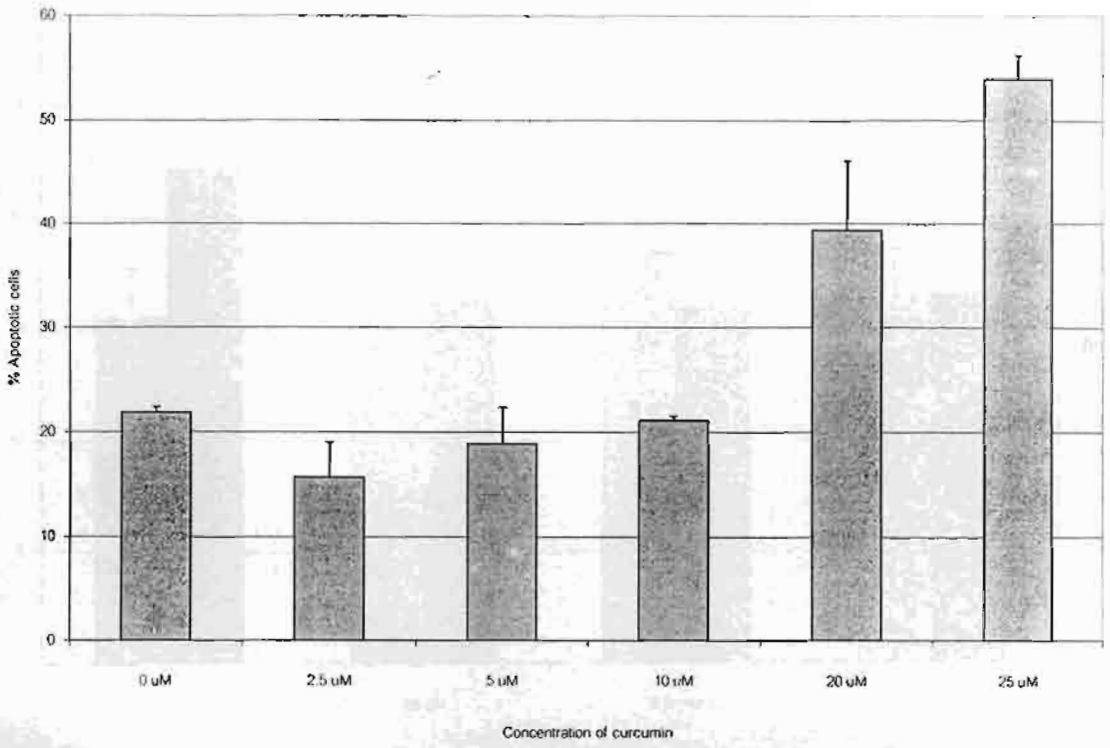


Fig. 7 The effect of curcumin on HL-60 cell apoptosis at various concentrations which was in a dose dependent manner.

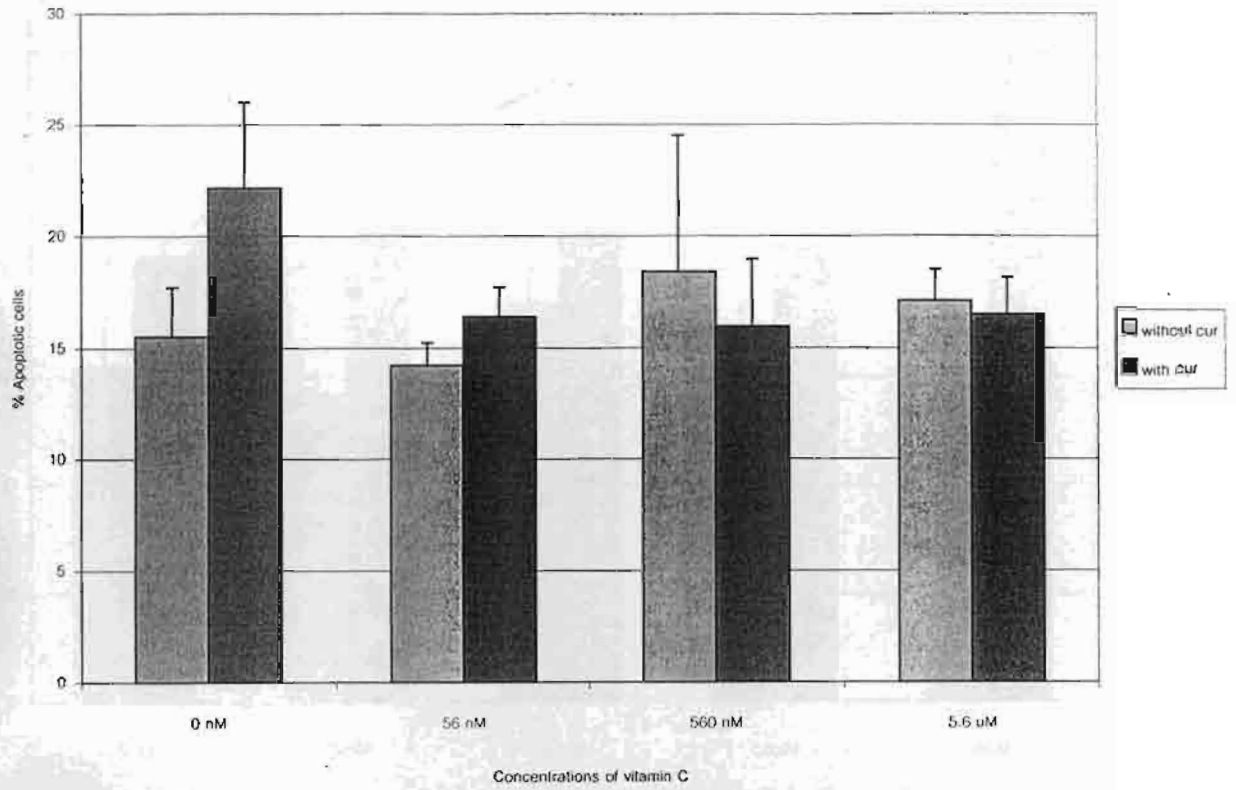


Fig. 8 The effect of vitamin C on HL-60 cell apoptosis induced by curcumin (10 μM) when treated simultaneously at various concentrations.

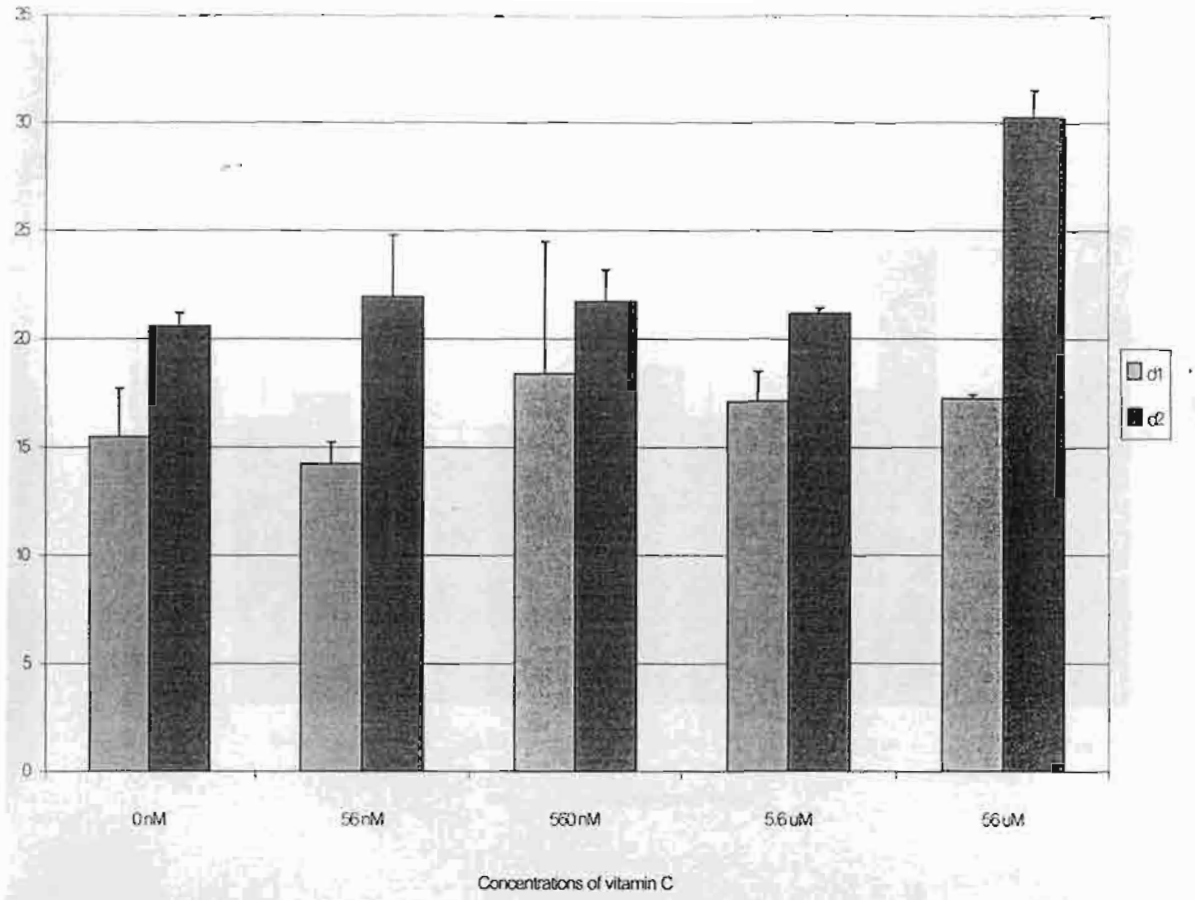


Fig. 9 The effect of vitamin C on HL-60 cell apoptosis on day 1 and day 2.

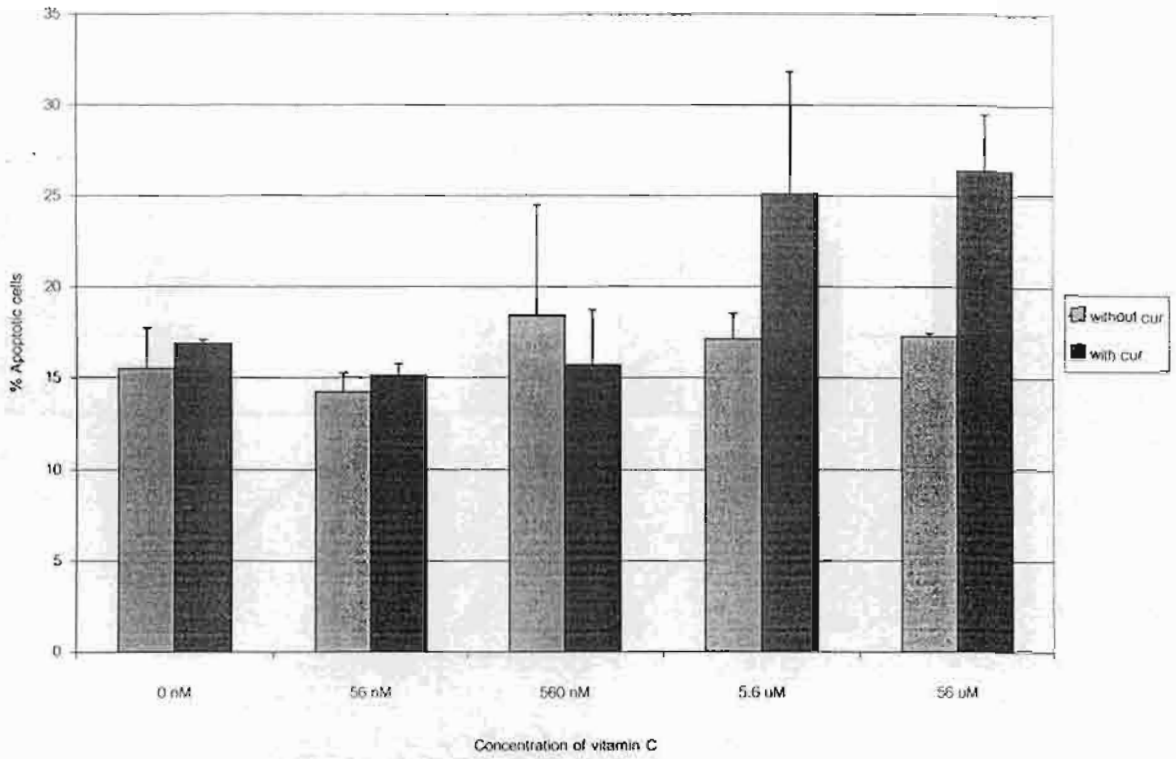


Fig. 10 The effect of vitamin C on HL-60 cell apoptosis treated for 1 day and then treated with curcumin (10 μM) for another day.

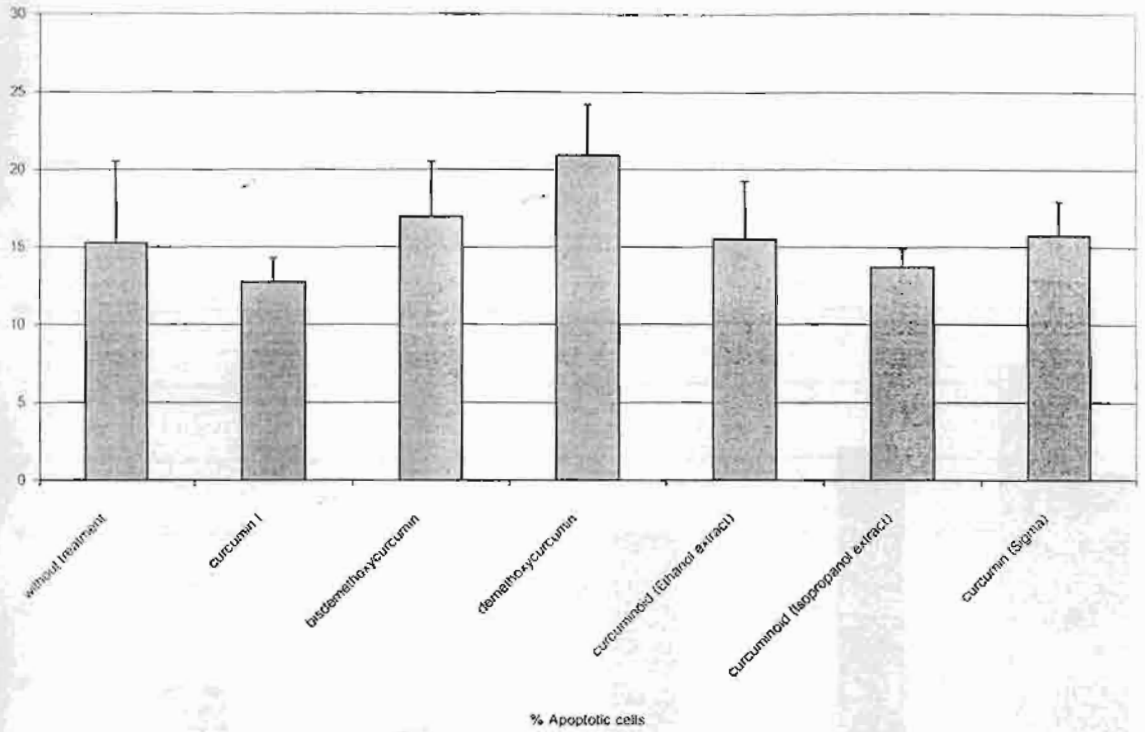


Fig. 11 The effect of curcuminoids (Ethanol extract and isopropanol extract) and curcumin derivatives on HL-60 cell apoptosis induced at the concentration of 10 μM.

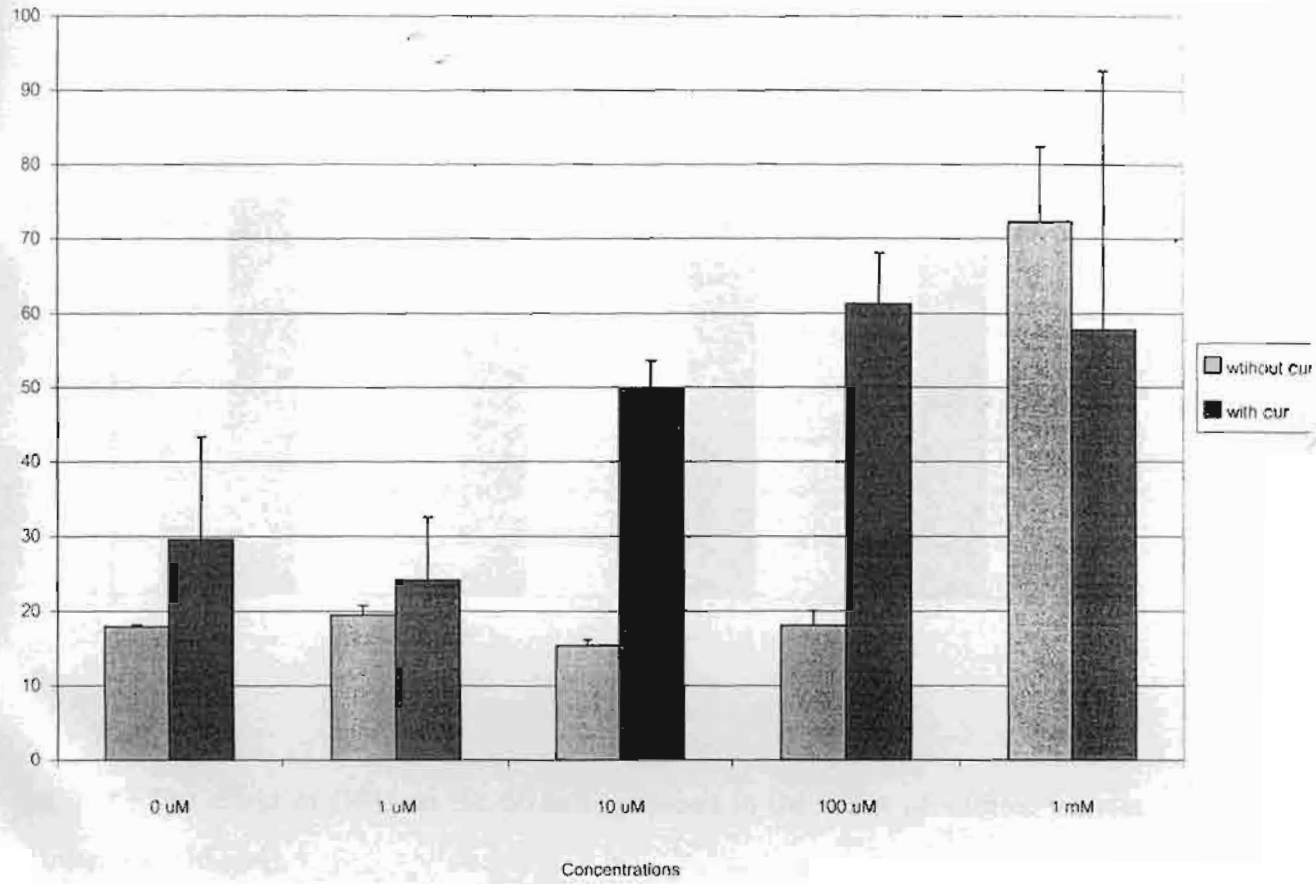


Fig. 12 The effect of Trolox on HL-60 cell apoptosis in the presence or absence of curcumin (10 μM).

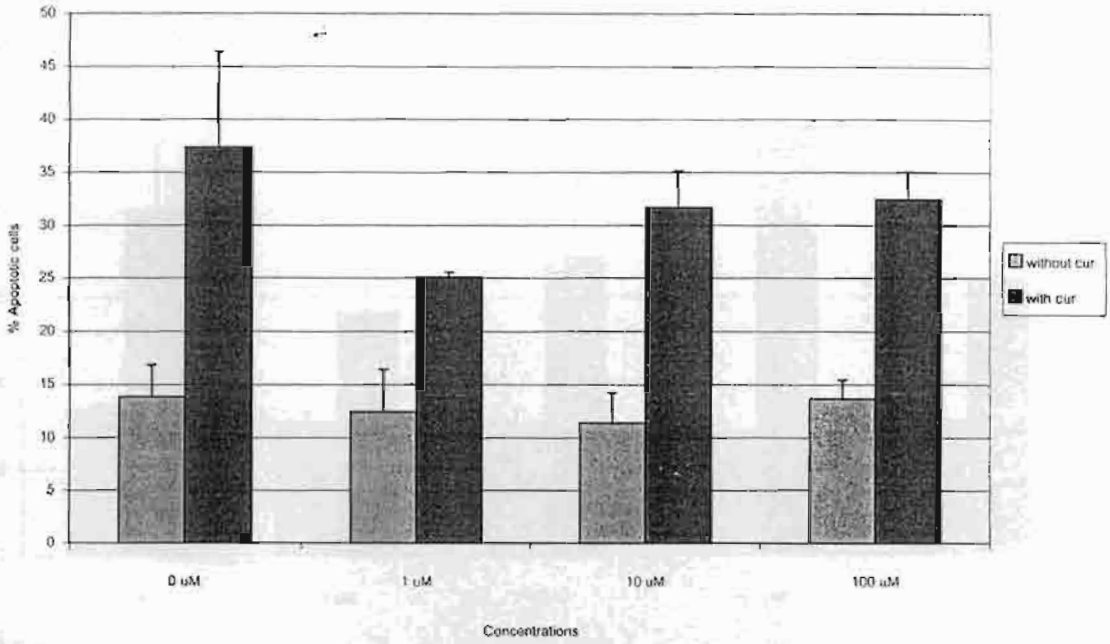


Fig. 13 The effect of GSH on HL-60 cell apoptosis in the status of with or without curcumin (10 μM).

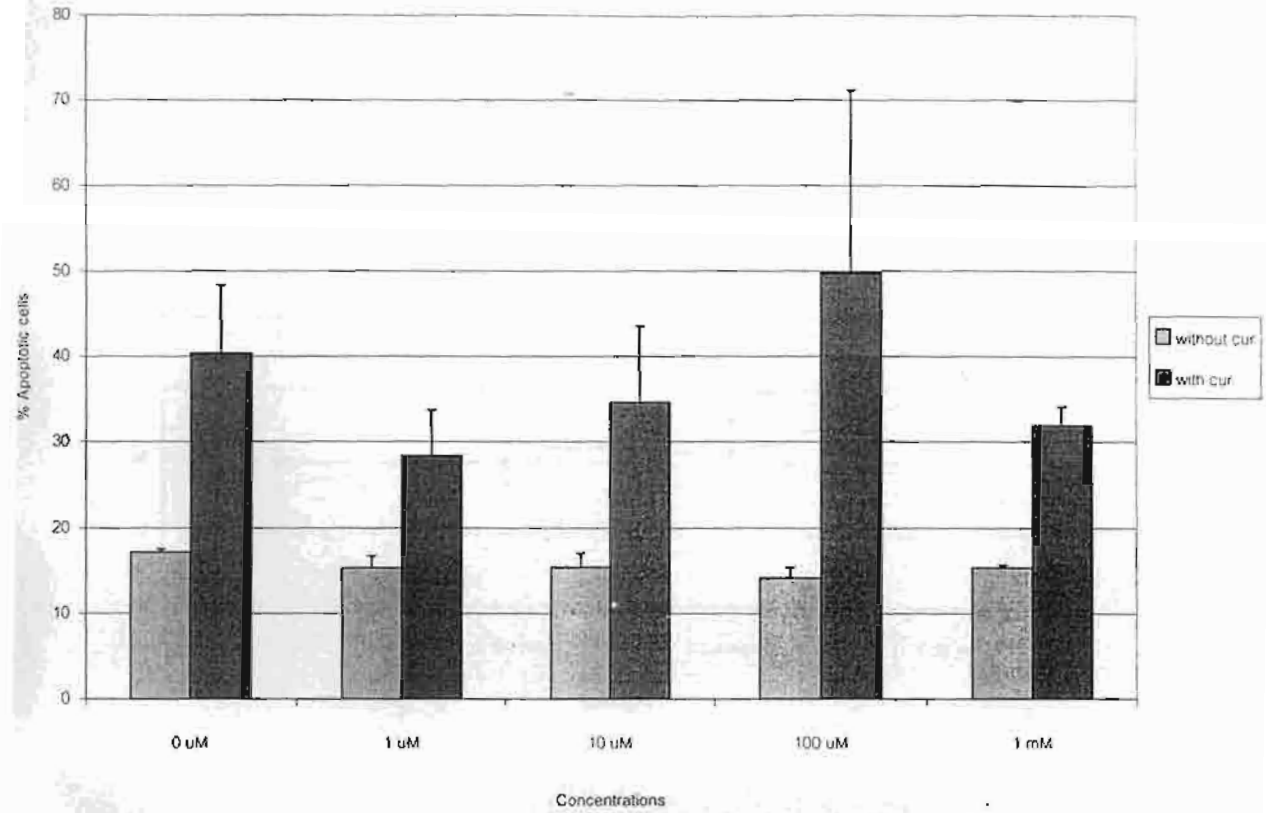


Fig. 14 The effect of N-acetylcysteine on HL-60 cell apoptosis in the status of with or without curcumin (10 μM).

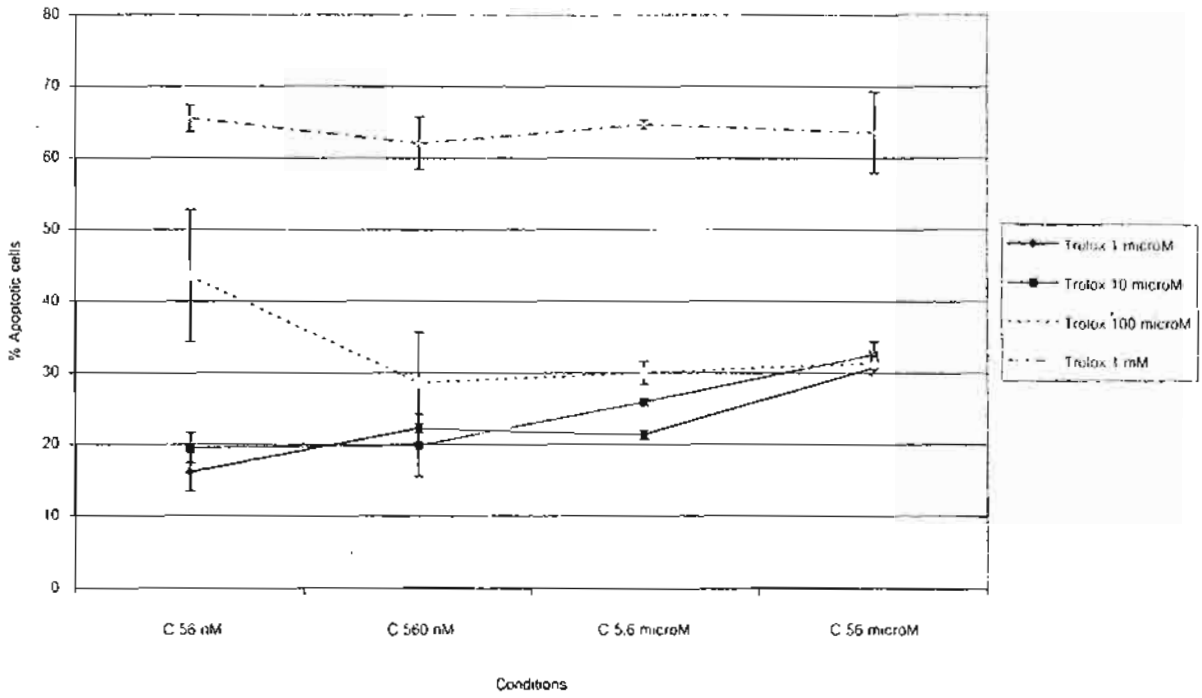


Fig. 15 The effect of vitamin C and Trolox on HL-60 cell apoptosis induced by curcumin at 10 μM.

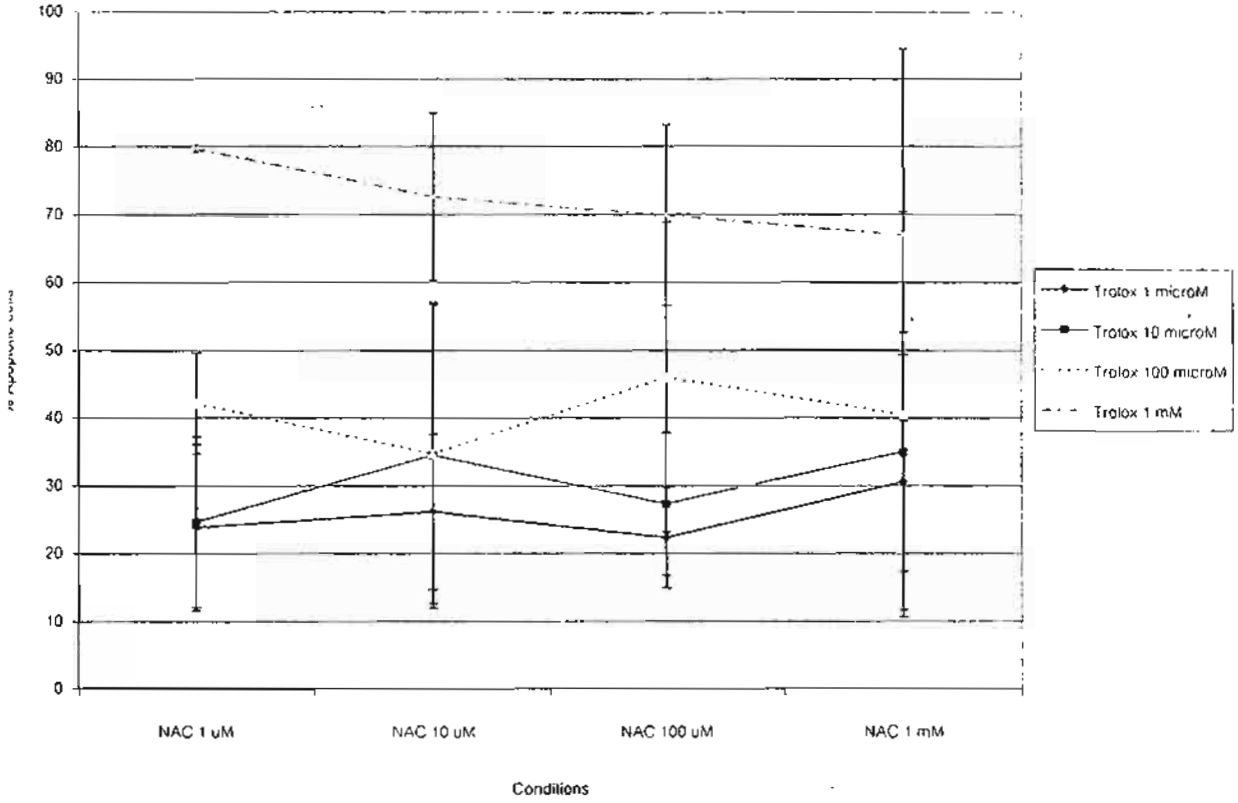


Fig. 16 The effect of Trolox and NAC on HL-60 cell apoptosis induced by curcumin at 10 μM.

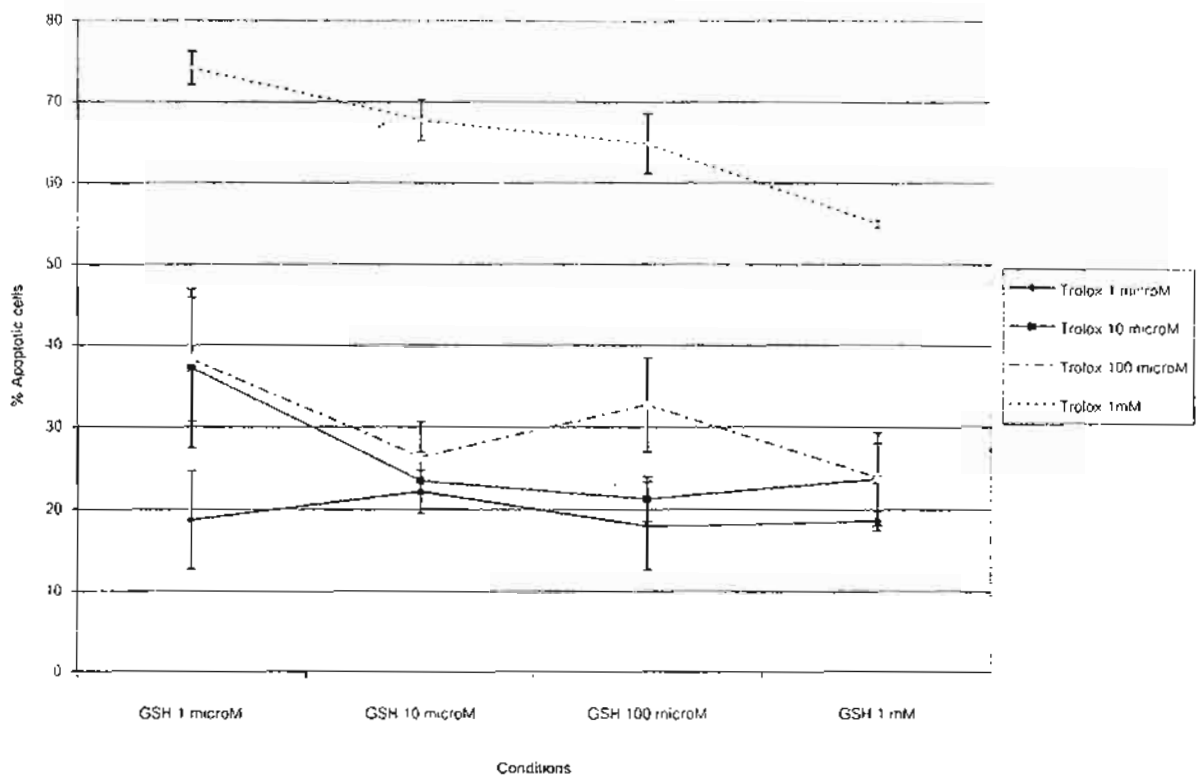


Fig. 17 The effect of Trolox and GSH on HL-60 cell apoptosis induced by curcumin at 10 μM.

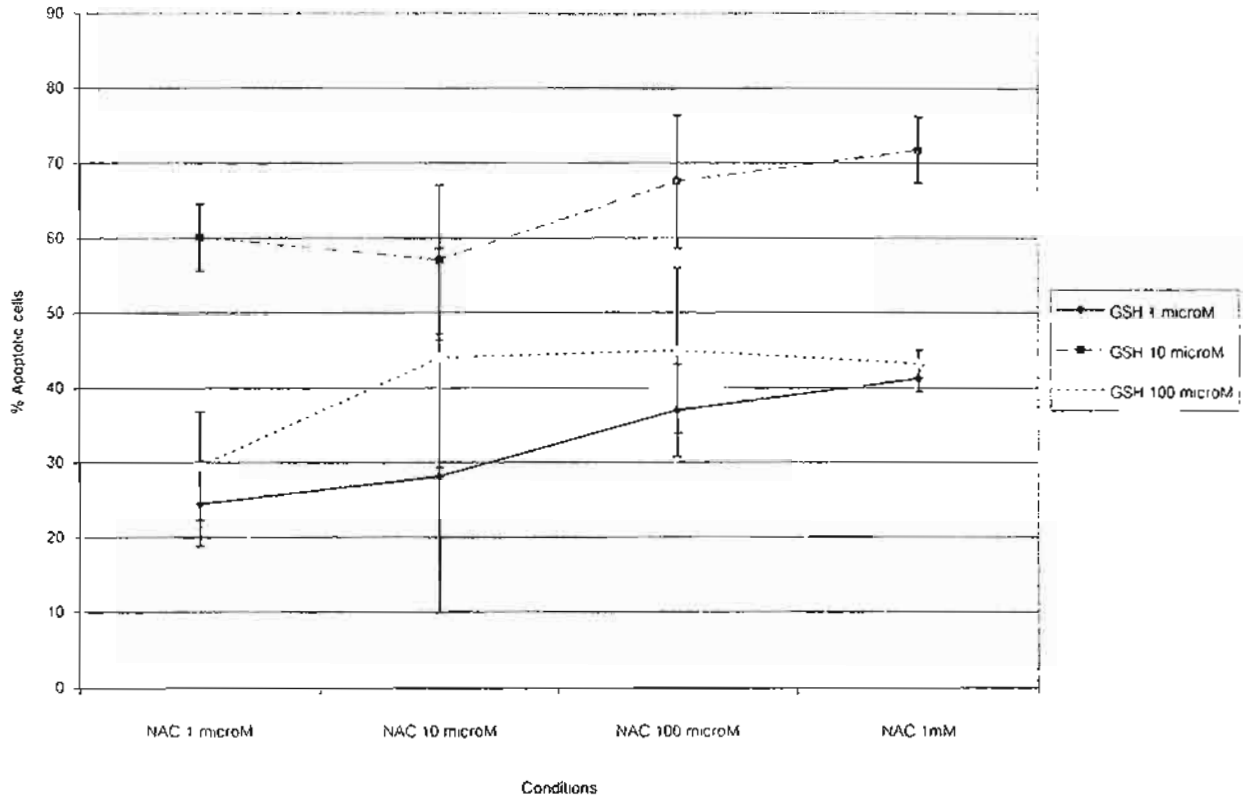


Fig. 18 The effect of GSH and NAC on HL-60 cell apoptosis induced by curcumin at 10 μM.

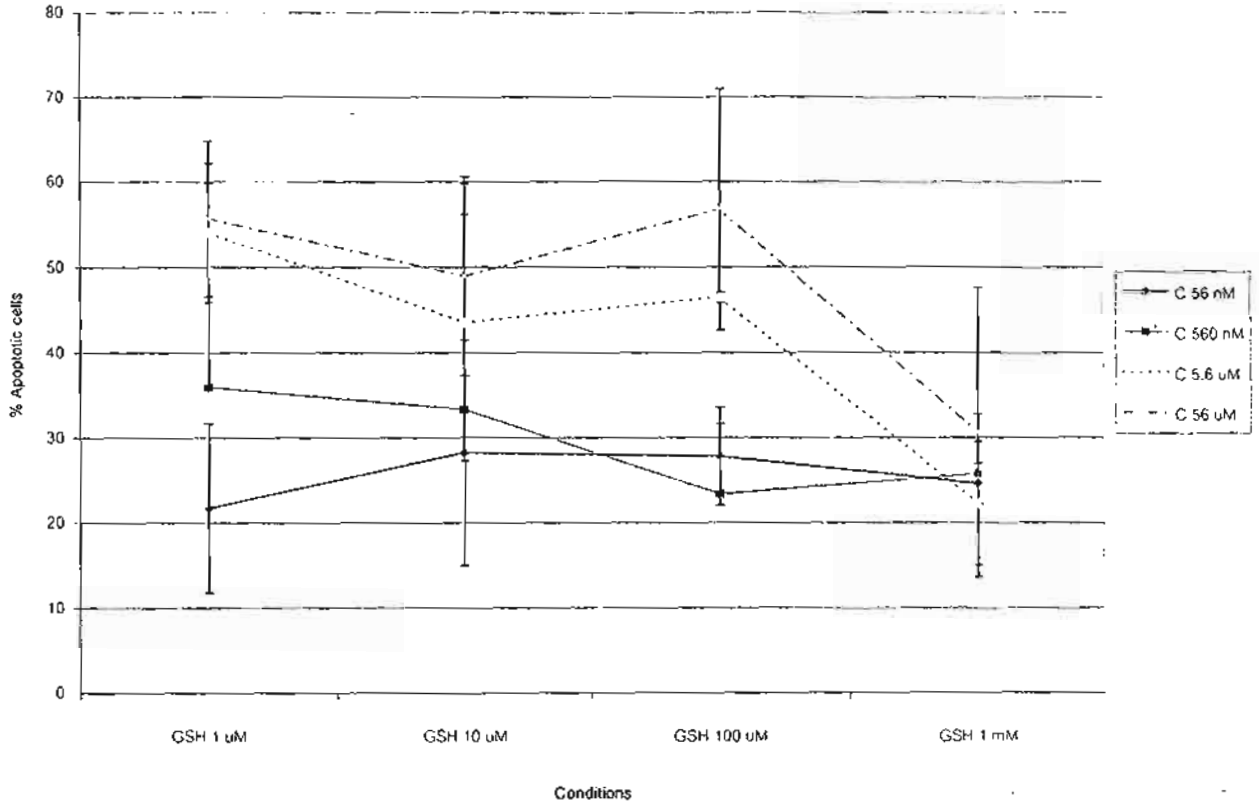


Fig. 19 The effect of vitamin C and GSH on HL-60 cell apoptosis induced by curcumin at 10 μM.

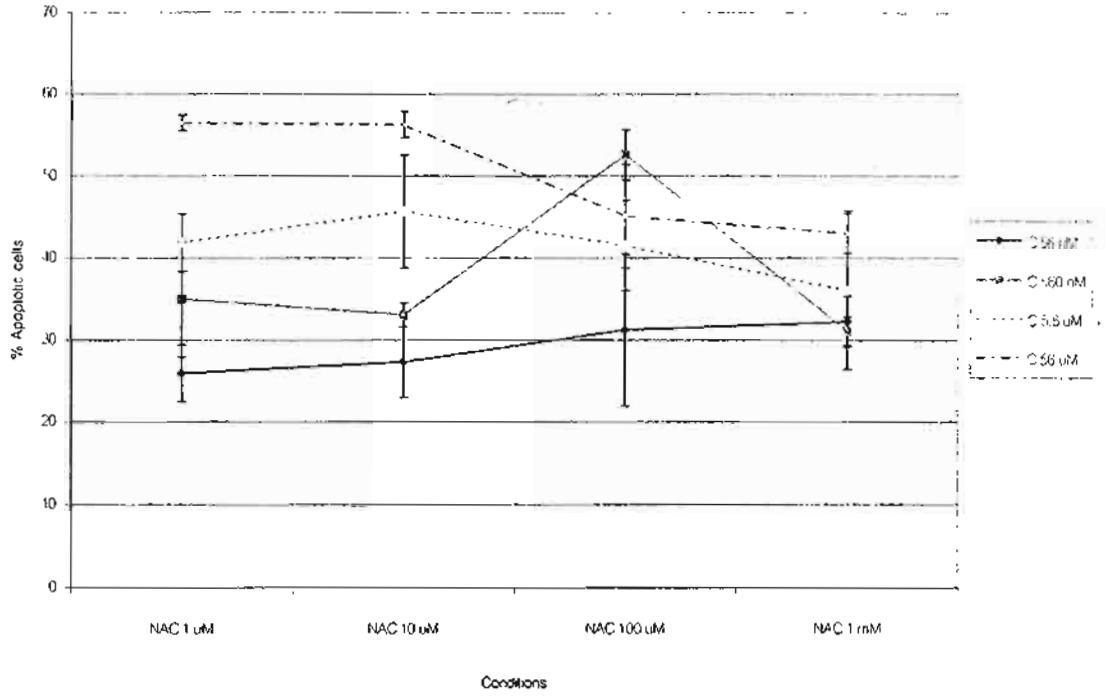


Fig. 20 The effect of vitamin C and NAC on HL-60 cell apoptosis induced by curcumin at 10 μM.

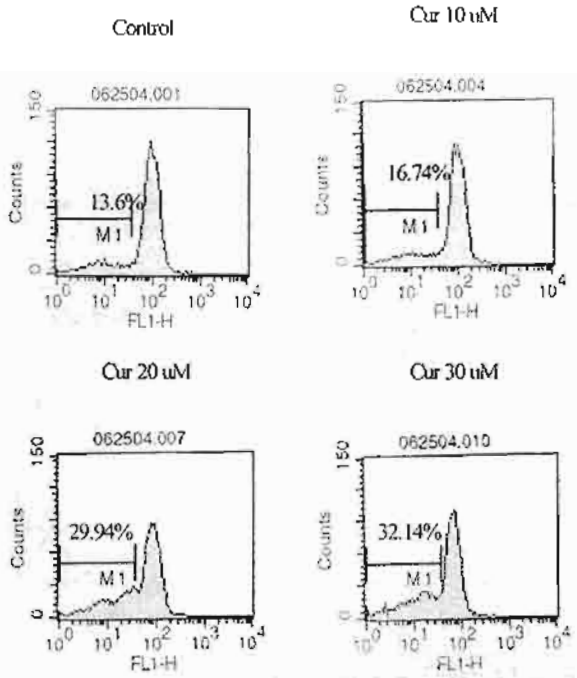


Fig. 21 Reduction of mitochondrial membrane potential when HL-60 cells were treated with curcumin at 0, 10, 20 and 30 μM for 4 h. Aliquots of $1x10^6$ cells were incubated with 50 nM 3,3'-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and analyzed by flow cytometry. The percentages reflect the reduction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

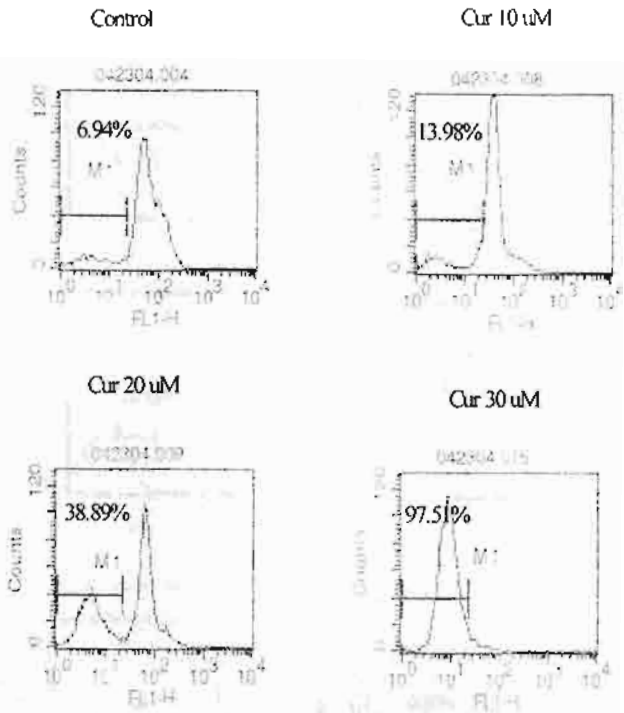


Fig. 22 Reduction of mitochondrial membrane potential when HL-60 cells were treated with curcumin at 0, 10, 20 and 30 μM for 24 h.

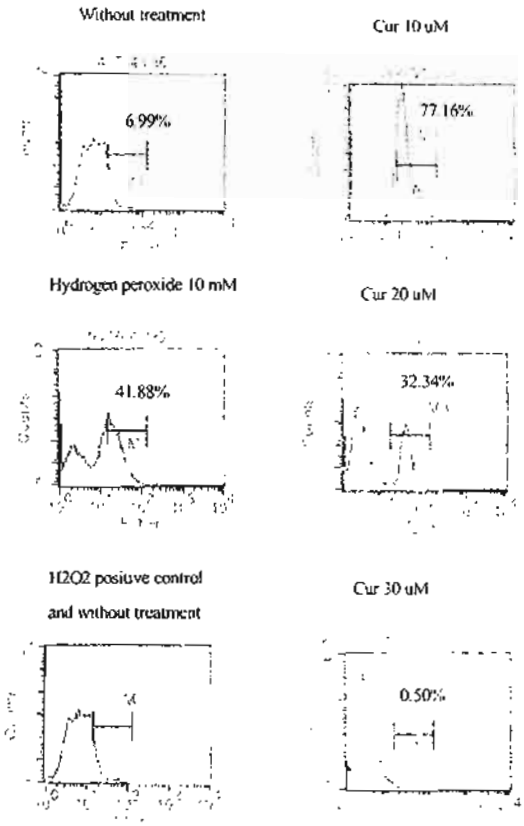


Fig. 23 The production of ROS (peroxides or DCFH) after curcumin treatment for 4 h. The open peak at left lower picture was a positive control after 10 mM hydrogen peroxide treatment (overlay).

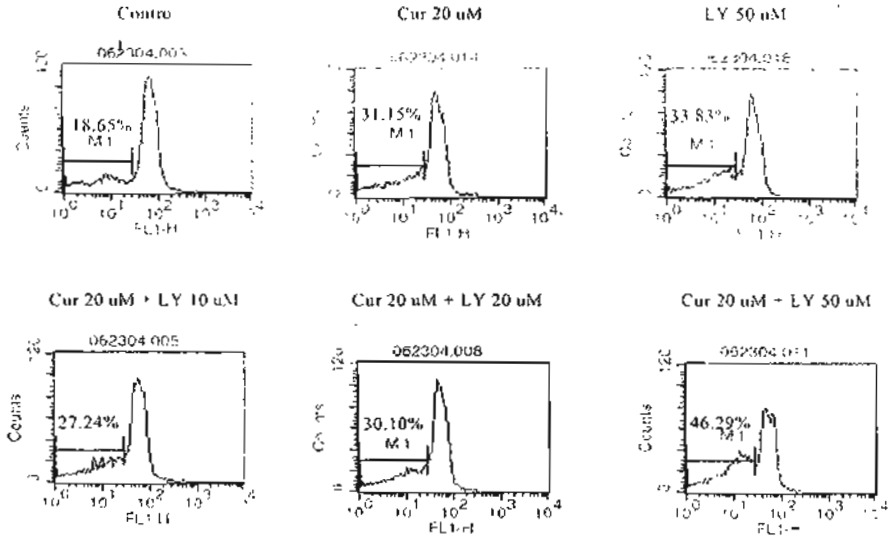


Fig. 24 The pattern of mitochondrial membrane potential when HL-60 cells were pretreated with LY294002 (PI3K inhibitor) at 10, 20 and 50 μM for 50 minutes and then with curcumin at 20 μM for 4 hours or with curcumin 20 μM alone or with LY294002 50 μM alone for 4 hours. Then $DiOC_6(3)$ was added at 40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

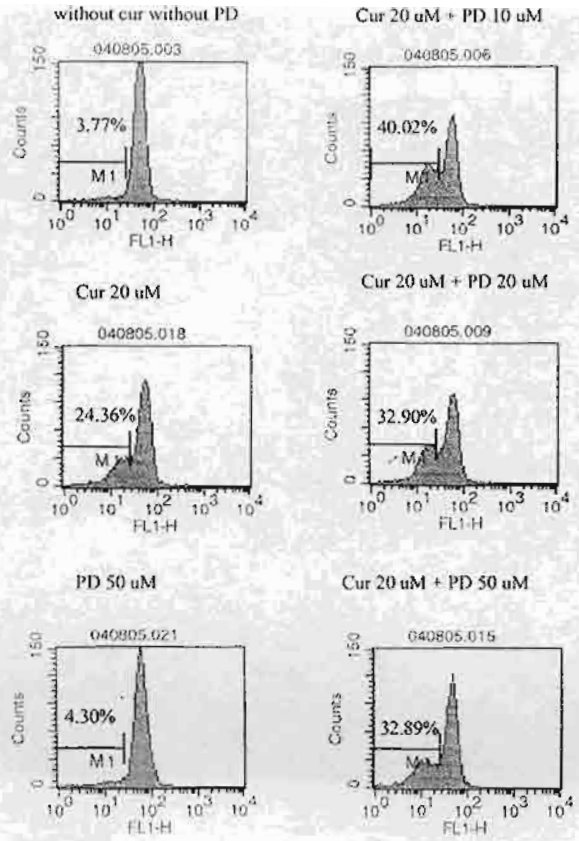


Fig. 25 The effect of PD98059 (MEK or MAP kinase kinase inhibitor) on HL-60 cells induced by curcumin. HL-60 cells were pretreated with PD98059 for 50 min and then incubated with curcumin (20 μM) for 4 hours. The HL-60 cells were treated with curcumin (20 μM) alone or PD98059 (50 μM) alone were compared.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

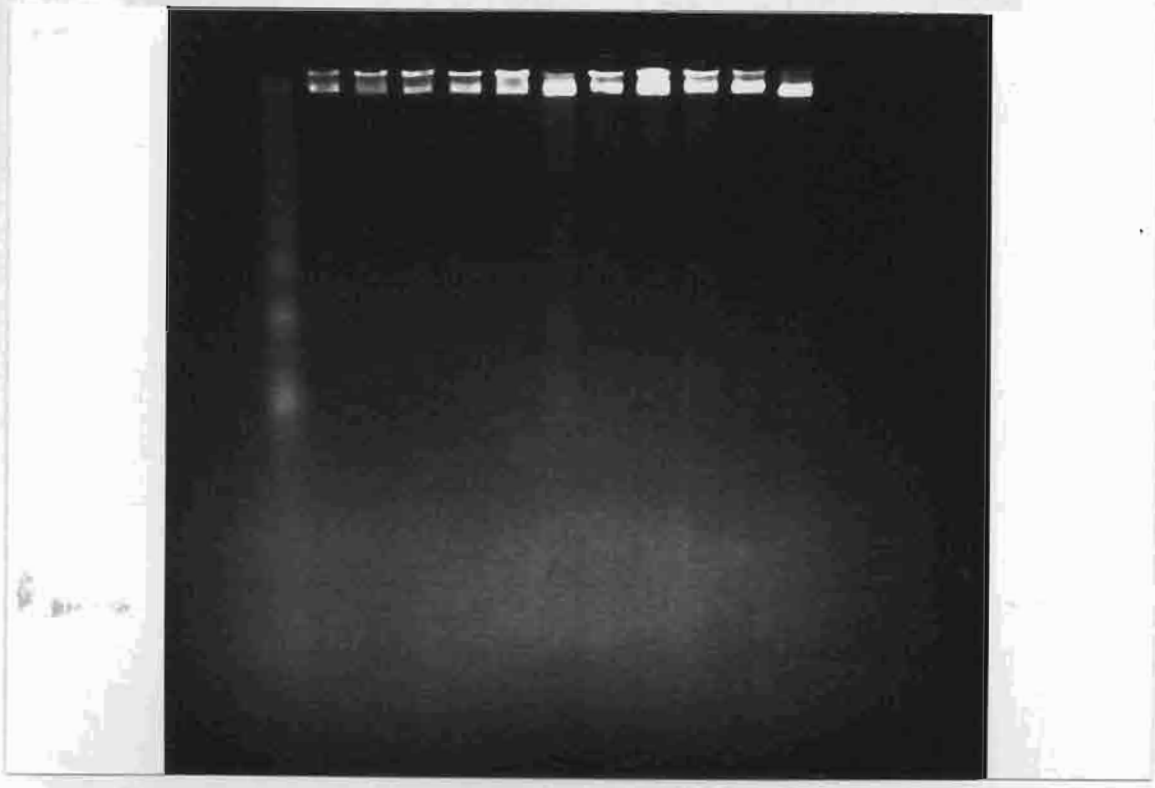


Fig. 26 Agarose gel electrophoresis pattern of HL-60 cells treated with curcumin. Lane 1 HL-60 cells treated with camtothecin 3 μM; Lane 2 control (without treatment); Lane 3 HL60 cells treated with curcumin 1 ng/ml day 1; Lane 4 with curcumin 10 ng/ml day 1; Lane 5 with curcumin 100 ng/ml day 1; Lane 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.

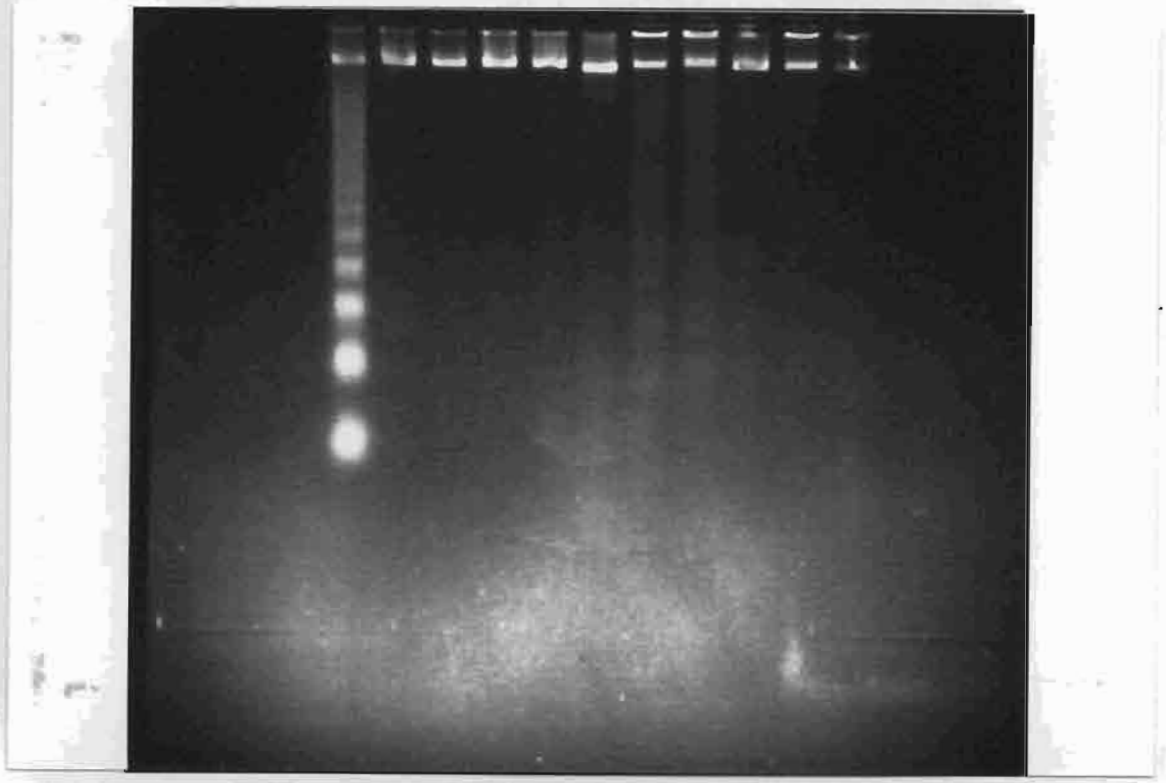


Fig. 27 Agarose gel electrophoresis pattern of HL-60 cells treated with curcumin. Lane 1 HL-60 treated with camtothecin; lane 2 control day 1; lane 3 curcumin 1 μg/ml day 1; lane 4 curcumin 2 μg/ml day 1; lane 5 curcumin 4 μg/ml day 1; lane 6 curcumin 8 μg/ml day 1; lane 7 control day 2; lane 8 curcumin 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.

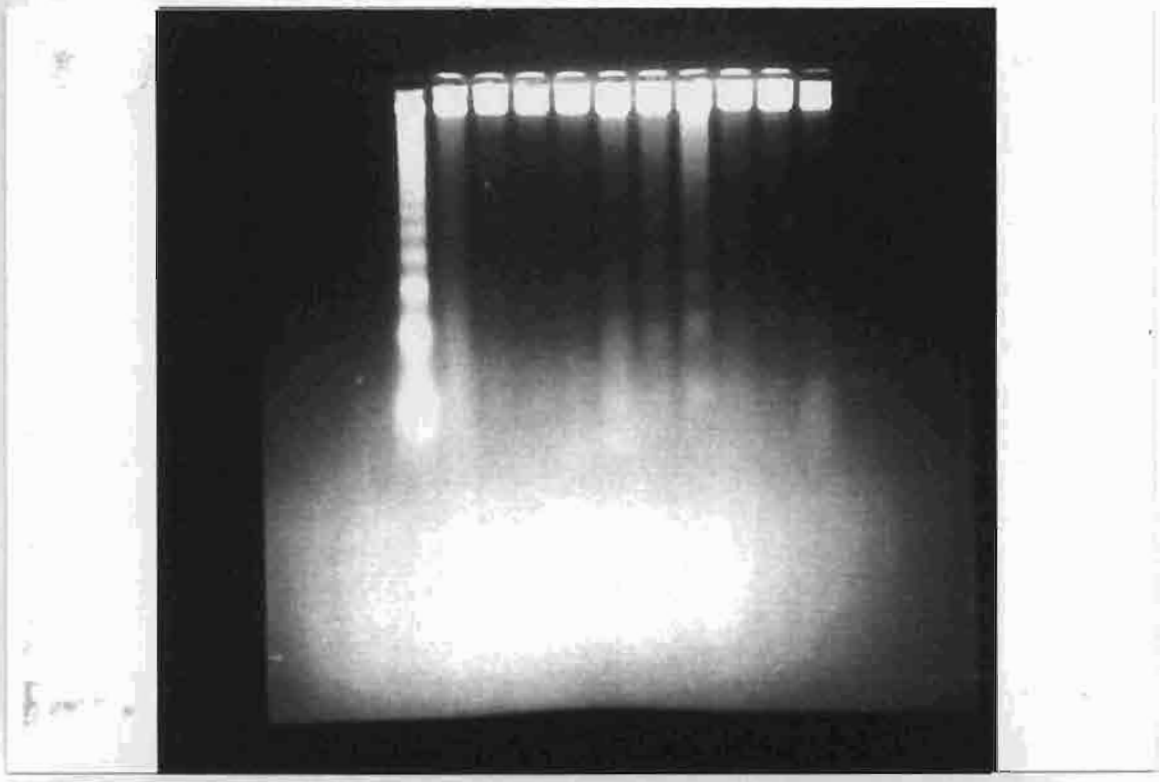


Fig. 28 Agarose gel electrophoresis pattern of HL-60 cells treated with curcumin. Lane 1 HL-60 cells treated with camtothecin; lane 2 control day 1; lane 3 curcumin 1 μg/ml day 1; lane 4 curcumin 2 μg/ml day 1; lane 5 curcumin 4 μg/ml day 1; lane 6 curcumin 8 μg/ml day 1; lane 7 control day 2; lane 8 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.

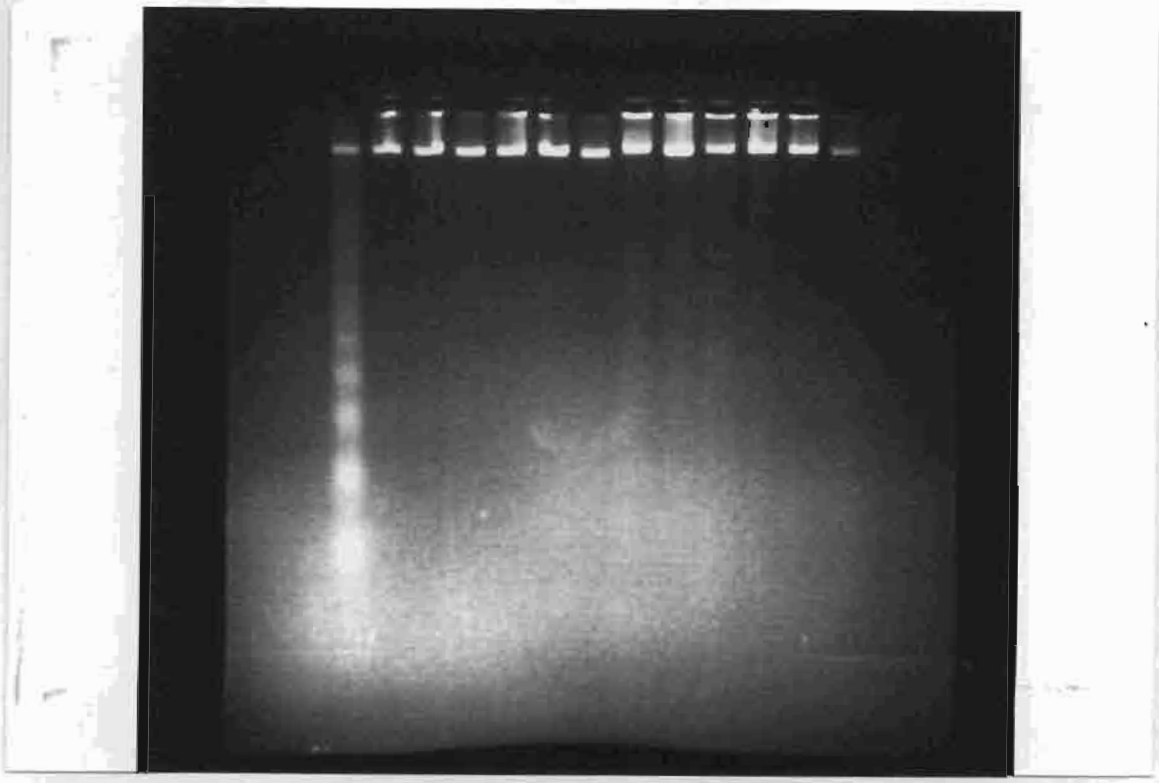


Fig. 29 Agarose gel electrophoresis pattern of HL-60 cells treated with curcuminoid extract (isopropanol) lane 1 HL-60 treated with camtothecin; lane 2 without treatment day 1; lane 3 curcuminoid (isopropanol extract) 1 ng/ml day 1; lane 4 curcuminoid (isopropanol extract) 10 ng/ml day 1; lane 5 curcuminoid (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.

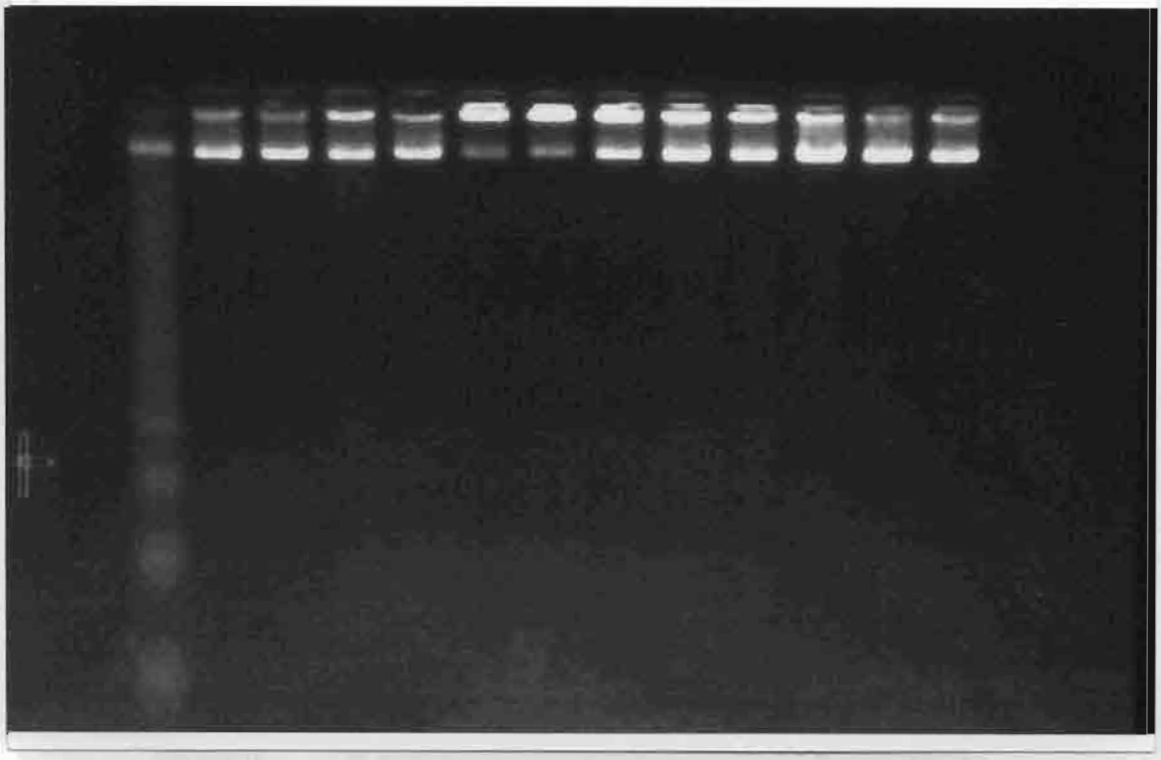


Fig. 30 Agarose gel electrophoresis pattern of HL-60 cells treated with curcuminoid (alcoholic extract). Lane 1 HL-60 cells treated with camtothecin; lane 2 control day 1; lane 3 curcuminoid (alcoholic extract) 1 ng/ml day 1; lane 4 curcuminoid (alcoholic extract) 10 ng/ml day 1; lane 5 curcuminoid (alcoholic 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.

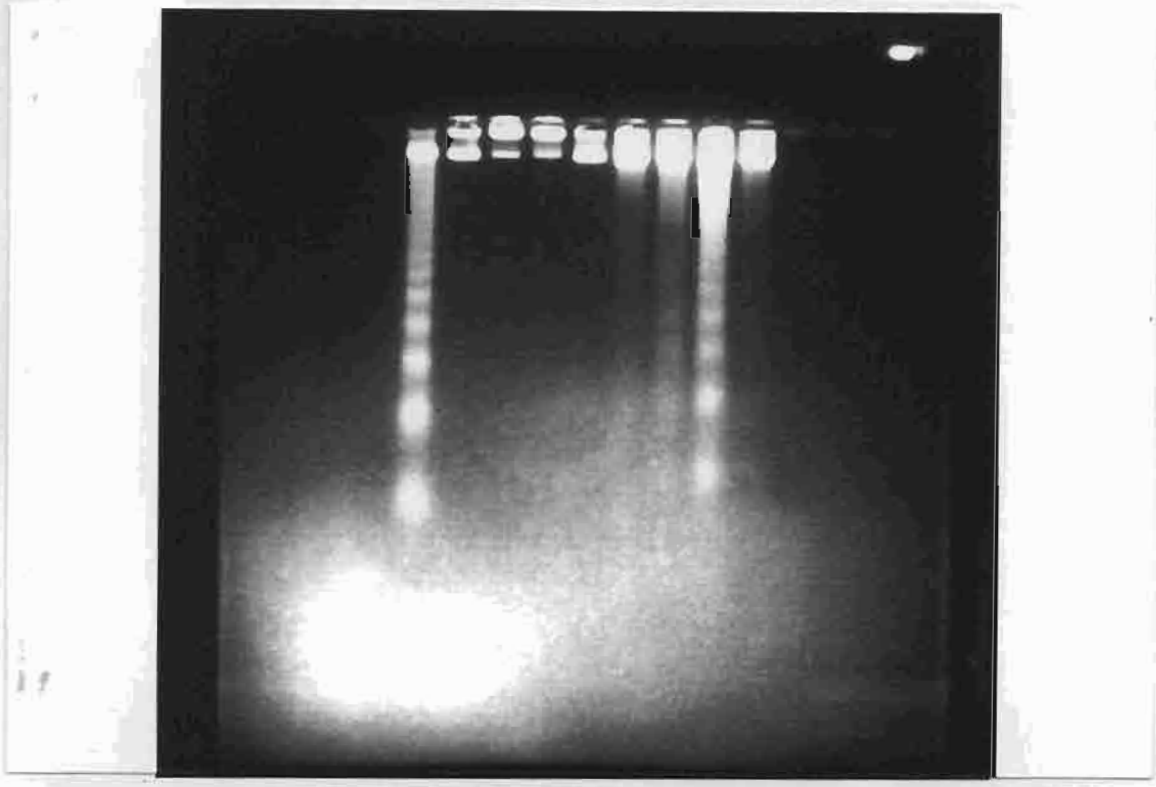


Fig. 31 Agarose gel electrophoresis pattern of HL-60 cells treated with hydrogen peroxide in media or PBS. Lane 1 HL-60 cells treated with camtothecin; lane 2 control in PBS; lane 3 HL-60 cells treated with 0.1 mM hydrogen peroxide in PBS; lane 4 HL-60 cells treated with 1 mM hydrogen peroxide in PBS; 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).