

Curcumin from Turmeric (*Curcuma longa*) Induced Apoptosis in Human Mammary Carcinoma Cells (MDA-MB-231)

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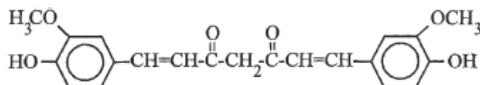
ABSTRACT

Introduction: Curcumin, a natural compound present in turmeric (*Curcuma longa*) has been known to possess both anti-inflammatory and antioxidant effects. **Objectives:** The objectives of the study were to evaluate the cytotoxic activities and to determine the mode of cell death induced by curcumin towards the human mammary carcinoma cells (MDA-MB-231). **Methodology:** Cytotoxicity of curcumin and its effect on cell viability were determined by using MTT assay and trypan blue dye exclusion method, respectively. The mode of cell death was detected by viewing under a light microscope and through DNA fragmentation analysis. **Results and discussion:** Curcumin was cytotoxic to MDA-MB-231 cells with the IC₅₀ of 17.25 μ g/ml. Cell viability treatment using curcumin at concentrations of 30 μ g/ml and 10 μ g/ml was significantly ($p < 0.05$) reduced at 48 and 56 hours, respectively, compared to the control. Cells treated with curcumin (30, 10, 3, and 1 μ g/ml) showed apoptotic features such as shrinkage of cell, membrane blebbing, chromatin condensation and nuclear fragmentation under a light microscope. DNA extracted from cells treated with curcumin at all concentrations were intact after 24 hours but fragmented after 72 hours, forming DNA laddering on agarose gel, a hallmark of apoptosis. **Conclusion:** Induction of apoptosis in breast cancer cells by curcumin suggests its potential use as a strategy for cancer control.

Keywords: Curcumin, *Curcuma longa*, cytotoxic, apoptosis

INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], with molecular chemical formula of C₂₁H₂₀O₆ is the major constituent and active ingredient of *Curcuma longa* (turmeric), a plant which belongs to the Zingiberaceae. Curcumin belongs to the class of curcuminoids.^[1]



Chemical structure of curcumin

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Curcumin exhibits a broad spectrum of effects, including anti-inflammatory, anti-mutagenic, anti-proliferative, and anti-carcinogenic activities.^[2] Curcumin has been shown to chemoprevent carcinogenesis^[3] and inhibit the growth and metastasis of tumour cells.^[4] Exposure of tumour cell lines to curcumin *in vitro* has resulted in inhibition of cell proliferation or induction of apoptotic cell death.^[5] In a previous report, curcumin was shown to inhibit the proliferation and cell cycle progression of human umbilical vein endothelial cells.^[6]

The objective of the study was to determine the *in vitro* cytotoxic properties and mode of cell death of curcumin towards the human mammary carcinoma (MDA-MB-231) cells.

MATERIALS AND METHODS

Compound

Curcumin was supplied by the Department of Chemistry, Faculty of Sciences, Universiti Putra Malaysia. The compound was dissolved in dimethylsulfoxide (DMSO) to give a stock concentration of 10 mg/ml.

Cells

The human mammary carcinoma (MDA-MB-231), acute myeloblastic leukemia (HL-60), breast adenocarcinoma (MCF-7), ovarian cancer (CaOV3), cervical epithelial carcinoma (HeLa) and Chinese hamster ovary (CHO) cell lines were purchased from the American Type of Culture Collection (ATCC), USA. The cells were grown in RPMI 1640 medium supplemented with 10% of fetal calf serum and antibiotics (100 IU of penicillin/100 mg/ml of streptomycin). The cells were maintained in a 25 cm² T-flask and incubated at 37 C under 5% CO₂ in a humidified atmosphere. Cell viability was measured by staining the cells with trypan blue and counted in a hemocytometer under a light microscope.

Determination of Cytotoxicity (MTT Assay)

Cytotoxicity assay was performed in a 96-well microtitration plate. The cells at an initial concentration of 1 x 10⁶ cells/ml were treated with 30, 10, 3, 1, 0.3, 0.1 and 0.03 µg/ml of curcumin. Initially, various concentrations of the compound were prepared from the substock solution by serial dilution in RPMI 1640 to give a volume of 100 µl in each of the microtiter-plate wells. Subsequently, 100 µl of the cell suspension was added into each well. Control without the compound was included. The assay was performed in triplicate. The plates were then incubated at 37 C, 5% CO₂, 90% humidity for 72 hours.

Cytotoxicity of curcumin was quantitatively estimated by a non-radioactive, colorimetric assay system using tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide). Briefly, MTT was dissolved in phosphate buffered saline at 5 mg/ml and filter-sterilised to remove a small amount of insoluble residue present in some batches of MTT. The MTT solution was added directly to all appropriate microtitre-plate wells (10 µl per 100 µl medium) containing cells, complete growth medium with or without the tested compound. The plate was then incubated for 4 hours at 37 C to allow MTT metabolism to formazan. Subsequently, the supernatant was aspirated and 100 µl of DMSO was added

and mixed thoroughly to dissolve the dark blue formazan crystals. Optical density (OD) was measured on an automated spectrophotometric EL 340 multiplate reader (Bio-Tek Instruments Inc., USA) using the test and reference wavelength of 570 and 630 nm, respectively. The cytotoxic concentration/dose that killed cells by 50% (IC₅₀) was determined from absorbance (OD) versus concentration curve.

Determination of Cell Viability

The MDA-MB-123 cells with a density of 1×10^5 cells/ml were treated with 30, 10, 3 and 1 $\mu\text{g/ml}$ of curcumin. Control (untreated cells) was included. The cells were harvested every 8 hours for 3 days (72 hours) and viability was measured by staining the cells with 0.25% trypan blue at a dilution of 1:1, and counted in a hemocytometer under a light microscope. The dead cells were stained blue while the viable cells remained clear. The percentage of cell viability can be calculated using the formula:

$$\text{Percentage of viability} = \text{number of viable cells} / \text{total cell number} \times 100$$

Determination of Morphological Changes

The untreated and curcumin-treated MDA-MB-123 cells (30, 10, 3 and 1 $\mu\text{g/ml}$) for 24 and 72 hours were viewed under an inverted light microscope. Apoptotic characteristics were identified by the appearance of cell shrinkage, nuclear condensation, and/or the presence of membrane-bound apoptotic bodies. Necrotic characteristics were identified by the appearance of irregular clumping of chromatin, swelling of all cytoplasmic compartments and focal disruption of membranes.

Determination of Mode of Cell Death (DNA Fragmentation Analysis)

DNA from the untreated and curcumin-treated MDA-MB-123 cells (30, 10, 3 and 1 $\mu\text{g/ml}$) for 24 and 72 hours were isolated using the Apoptotic DNA Ladder Detection Kit (Chemicon, USA) and the manufacturer's established methods. Agarose gel electrophoresis was carried out for the analysis of fragmentation of DNA. GeneRuler™ 100 bp DNA Ladder Plus was used as a marker for size comparison. The agarose gel was then stained with ethidium bromide and DNA was visualised by UV illumination.

RESULTS

Cytotoxicity of Curcumin

The cytotoxic effects of curcumin were evaluated against a panel of cancerous cell lines inclusive of the human mammary carcinoma (MDA-MB-231), acute myeloblastic leukemia (HL-60), breast adenocarcinoma (MCF-7), ovarian cancer (CaOV3) and cervical epithelial carcinoma (HeLa) by using the MTT assay. Chinese hamster ovary (CHO) cell line was used as a representative of a normal cell line. Curcumin was found to be most cytotoxic to the human mammary carcinoma (MDA-MB-231) with the IC₅₀ value (a concentration that reduces the cell number to 50% as compared to the untreated value) at 17.25 $\mu\text{g/ml}$ (Table 1).

Table 1. Cytotoxicity of curcumin against cancerous and non-cancerous cell lines based on the IC_{50} value determined by the MTT assay

Cell line	IC_{50} (μ g/ml)
Human mammary carcinoma (MDA-MB-231)	17.25
Human cervical epithelial carcinoma (HeLa)	27.81
Human breast adenocarcinoma (MCF-7)	>30
Human ovarian cancer (CaOV3)	>30
Human acute myeloblastic leukemia (HL-60)	>30
Chinese hamster ovary (CHO)	>30

Effects of Curcumin on Cell Viability of MDA-MD-231 Cells

The viability of cells treated with 30 and 10 μ g/ml of curcumin was significantly reduced ($p < 0.05$) at 48 and 56 hours, respectively, compared to the control. There was a reduction, but not significant, in cell viability of treatments of lower concentrations of the compound (3 and 1 μ g/ml) (Fig. 1).

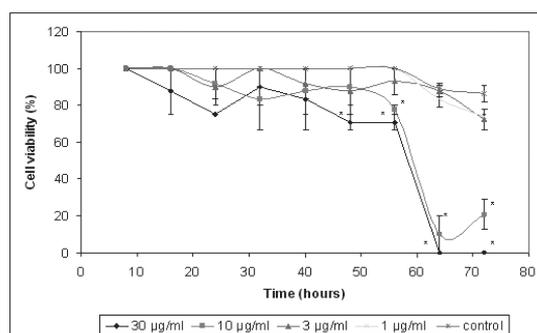


Figure 1. Effects of curcumin on cell viability. Cell viability was measured by staining the cell with trypan blue and counting in a haemocytometer under a light microscope. Results are expressed as mean \bar{x} SEM in triplicate determination. Differences between the treated and control were determined by the Independent-sample *T*-test.
* $p < 0.05$

Effects of Curcumin on Cell Morphology in MDA-MD-231 Cells

Unaffected normal viable MDA-MB-231 cells were fibroblast-like and were attached to the surface of the flask. The dead cells were detached from the surface and appeared oval or round in shape. They exhibited a few morphological features characteristic of apoptosis such as nuclear compaction, cytoplasmic constriction and a reduction in cell volume, membrane blebbing, and formation of apoptotic bodies. Necrotic cells were not observed (Fig. 2). The number of cells of all the treated groups was obviously fewer than the control. The number of non-viable cells was prominent in the treatment of the highest

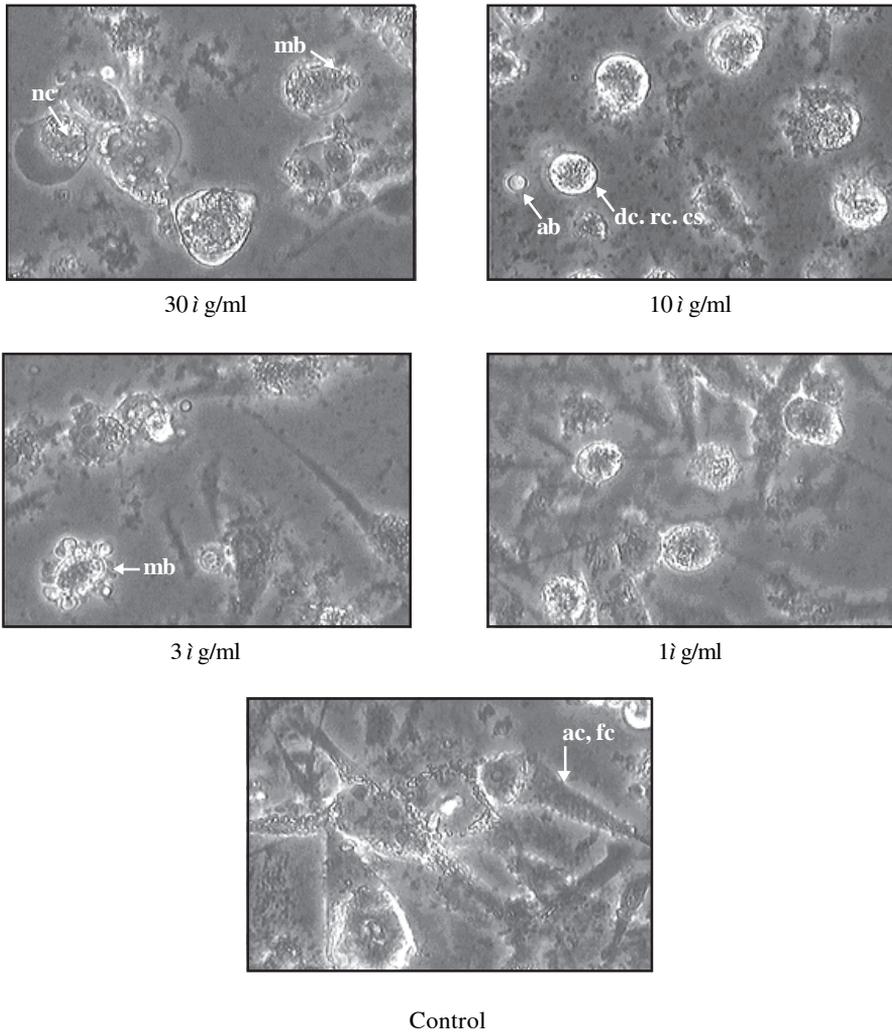


Figure 2. Effects of different concentrations of curcumin on morphology of MDA-MB-231 cells after 24 hours. Dead cells were rounded (rc) and detached from surface of the flask (dc). They exhibited some features of apoptosis such as nuclear condensation (nc), membrane blebbing (mb), cell shrinkage (cs) and formation of apoptotic bodies (ab). The unaffected healthy fibroblast-like cells (fc) remained attached (ac) to the surface of the flask (200X magnification).

of the highest concentration of curcumin (30 μ g/ml). In general, the number of attached cells (normal viable healthy cells) decreased as the concentration of the compound increased (Fig. 3).

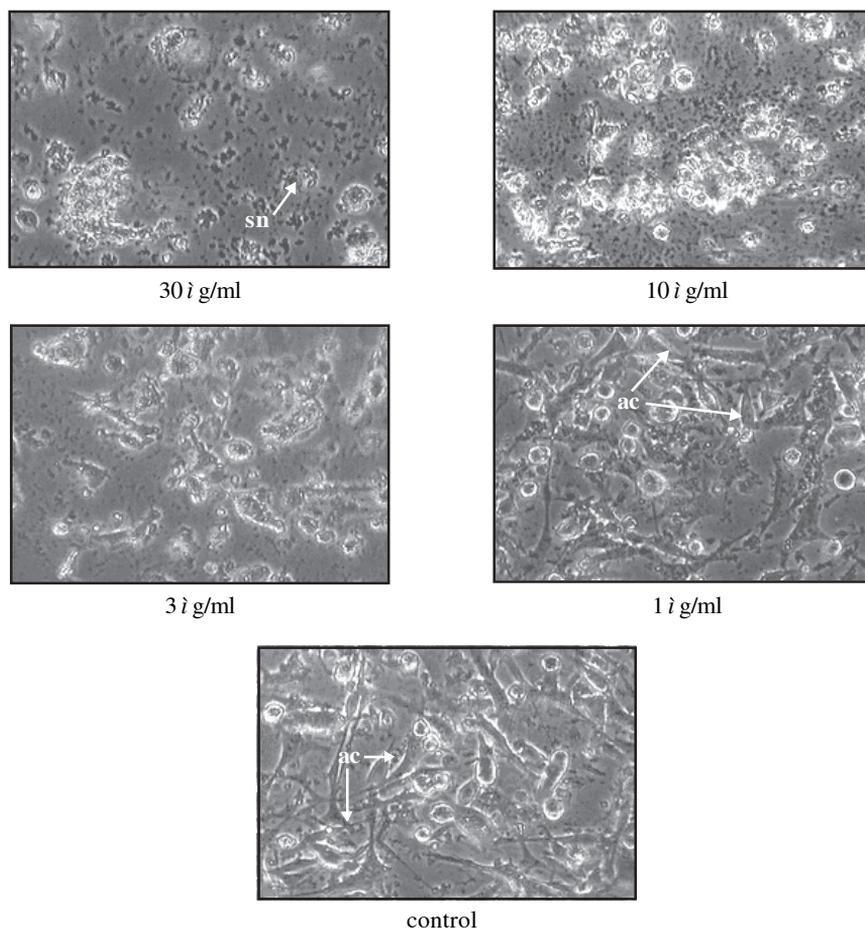


Figure 3. Effects of different concentrations of curcumin on morphology of MDA-MB-231 cells for 72 hours. The majority of the cells at treatment of 30 and 10 μ g/ml of curcumin underwent secondary necrosis (sn). Cell density decreased with the increase in concentration of curcumin. Number of attached fibroblast-like cells (ac) was fewer in the treated cells compared to the control (100X magnification).

Effects of Curcumin on DNA Fragmentation in MDA-MD-231 Cells

DNA patterns of the curcumin-treated and untreated cells were observed by the DNA fragmentation analysis. DNA from cells treated with all concentrations of the compound (30, 10, 3 and 1 μ g/ml) was intact after 24 hours (data not shown). After 72 hours, distinctive ladder-like pattern of DNA due to fragmentation that produced multiples of 200 base pairs was clearly observed on the agarose gel from treatments using 30, 10 and 3 μ g/ml of curcumin. Slight DNA degradation was observed from treatment of 1 μ g/ml of curcumin. DNA from the control remained intact (*Fig. 4*).

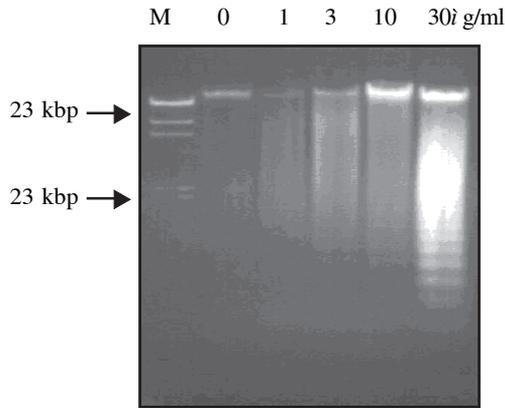


Figure 4. Effects of different concentrations of curcumin on DNA of MDA-MB-231 cells for 72 hours. The distinctive ladder-like pattern was observed from treatments of 30, 10 and 3 μ g/ml of curcumin. DNA of the control was intact. Lane M: Marker (HindIII digest of lambda DNA).

DISCUSSION

Curcumin was cytotoxic to a few cancerous cell lines used in the study. The human mammary carcinoma cell line (MDA-MB-231) was found to be most sensitive towards curcumin with the IC_{50} value of 17.25 μ g/ml (Table 1). The compound has been shown previously to be cytotoxic to some of the cell lines used in this study such as acute myeloblastic leukemia (HL-60), breast adenocarcinoma (MCF-7) and cervical epithelial carcinoma (HeLa), and also to chronic myelogenic leukemia (K-562).^[7] Nevertheless, the response was quite different from our findings, whereby curcumin was indicated to be most cytotoxic towards HL-60, K-562 and MCF-7 but did not show much activity in HeLa cells. This is possibly due to batch-to-batch variation in the cell lines used.

Cytotoxic effects of curcumin were time- and dose-dependent as it caused significant reduction in cell viability of MDA-MB-231 at higher concentrations (30 and 10 μ g/ml) and at different points of exposure (48 and 56 hours) (Fig. 1). An increase in cell viability indicates cell growth, while a drop in viability can be interpreted as a result of either toxic effects of compounds or suboptimal conditions. It shows that the higher the concentration of curcumin used and the longer it is exposed to the cells, the more toxic it will be. Shao *et al.*^[8] reported that the cytotoxic effects of curcumin towards MDA-MB-231 appear to be mediated through the downregulation of MMP-2 (matrix metalloproteinase) and the upregulation of TIMP-1 (tissue inhibitor of metalloproteinase), two common effector molecules that have been implicated in regulating tumour cell invasion.

MDA-MB-231 cells treated with curcumin at concentrations of 30, 10 and 3 μ g/ml rounded up, detached from the surface of the flask and shrunk. They exhibited some characteristics of apoptosis such as nuclear compaction, cytoplasmic constriction and

reduction in cell volume, membrane blebbing, and formation of apoptotic bodies (*Fig. 2*). The number of attached viable cells decreased with an increase in concentration of curcumin (*Figs. 2 and 3*). During apoptosis, cells initially round up and detach from the flask and their neighbours with subsequent condensation of cytoplasm, dissolution of the nuclear envelope and separation of the nucleus into distinct fragments, that ultimately brings to the collapse of the cell into several small intact vesicles (apoptotic bodies).^[9] The remarkable phenotypic alterations of apoptotic cells are caused by the destruction of the normal nuclear architecture and cleavage of the chromatin during the cell demolishing process. Collapse of the nucleus is thought to be due to destabilisation of the nuclear envelope as a consequence of lamin proteolysis. This results in lost of the matrix attachment region-points at which the chromatin is attached to the nuclear envelope, causing the chromatin to compact.^[10]

The induction of apoptosis in the MDA-MB-231 cells was further confirmed at molecular level by using the DNA fragmentation analysis. After 24 hours, the DNA was intact. A ladder-like pattern of DNA was visible on agarose gel from the treatments of 30, 10 and 3 μ g/ml of curcumin after 72 hours (*Fig. 4*). The ladder-like pattern is one of the hallmarks of apoptosis.^[9] The DNA fragmentation is caused by the activation of an endogenous nuclear endonuclease, which selectively, distinctively, cleaves the double-stranded nuclear DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligo-nucleosomal DNA fragments. These DNA fragments reveal discrete multiples of an approximately 180bp subunit. The degraded DNA produces a so-called ladder-like pattern when run on DNA agarose gel electrophoresis.^[11, 12, 13, 14] It has been suggested that DNA digestion in apoptotic cells is an ordered process of multiple progression, which may involve the collaborative action of a number of endonucleases and proteases, and that the nature of the endonucleases activated may be cell type specific.^[10]

CONCLUSION

Based on this study, it is concluded that curcumin is cytotoxic in a dose- and time-dependent manner to the human mammary carcinoma (MDA-MB-231) cells via an apoptosis-dependent pathway. Its action as inducer of apoptosis in the breast cancer cells suggests its potential use as a strategy for cancer control. This is due to the fact that apoptosis is a discrete manner of cell death that differs from necrotic cell death. Apoptosis is always regarded as an ideal way to destroy damaged cells, and an agent that can induce apoptosis is more preferable for the management and therapy of cancer.

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