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The antiproliferative and cytotoxic effects of curcumin on human cervical cancer Hep2C cell line

Kurkuminin insan servikal kanseri Hep2C hücre hattı üzerindeki antiproliferatif ve sitotoksik etkileri

Filiz SUSUZ ALANYALI[1] (ID), Mehmet ALKAN[2] (ID)

ABSTRACT

Objective: In this study, antiproliferative and cytotoxic effects of different concentrations of curcumin on cervical cancer Hep2C cells were investigated with microscopic methods and MTT assay.

Methods: Hep2C (human carcinoma cancer cell line, ATCC:CCL-23) cells were cultured. For cytotoxicity evaluation Hep2C cells exposed to curcumin at different concentrations of 30 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.7 µg/ml, 1.9 µg/ml, 0.9 µg/ml, 0.45 µg/ml for 24 hours These Hep2C cells are evaluated with MTT assay. The IC_{50} value of the agent for 24 h of exposure was detected. The graph of the absorbance data obtained by the Spectramax I3 device. Viability values of Hep2C cells calculated from the absorbances obtained from MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay are gained. The preparations were observed based on changes in nuclei and structures using an inverted microscope (Leica Microsystems). Nontreated cells were used as negative control and for positive control Hep2C cells were exposed to ammonium molibdate (1mg/ml) for the above given incubation period.

ÖZET

Amaç: Bu çalışmada, kurkuminin servikal kanser hücresi Hep2C üzerindeki antiproliferatif ve sitotoksik etkisi mikroskobik yöntemlerle ve MTT testi aracılığı ile araştırılmıştır.

Yöntem: Hücre kültüründe çoğaltılan Hep2C (insan karsinoma kanser hücre hattı, ATCC:CCL-23) hücreleri, farklı kurkumin konsantrasyonlarına; 30 µg/ml, 15 µg/ml, 7,5 µg/ml, 3,7 µg/ml, 1,9 µg/ml, 0,9 µg/ml, 0,45 µg/ml, 24 saat boyunca maruz bırakılmıştır. Bu hücreler MTT protokolü uygulanarak test edilmiştir. 24 saatlik maruz bırakılmadan sonra Spectramax I3 cihazıyla absorbans değerlerine dayalı grafik ve IC_{50} değeri elde edilmiştir. MTT (3-[4,5-dimetiltiyazol-2-il]-2,5-difenil-tetrazolyum bromür) testine dayalı olarak Hep2C hücrelerinin canlılık oranları tespit edilmiştir. Bu hücrelerdeki çekirdek ve yapısal değişiklikler inverted mikroskopla gözlemlenmiştir (Leica Microsystems). İşlem görmemiş hücreler negatif kontrol olarak kullanılmış ve pozitif kontrol için Hep2C hücreleri, yukarıda verilen inkübasyon süresi boyunca amonyum molibdata (1 mg/ml) maruz bırakılmıştır.

[1]Eskisehir Technical University, Faculty of Science, Department of Biology, Eskişehir
[2]Turkish Medicines and Medical Devices Agency, Turkish Ministiry of Health, Ankara

İletişim / Corresponding Author : Filiz SUSUZ ALANYALI
Eskişehir Teknik Üniversitesi Fen Fakültesi Biyoloji Bölümü 26470, Eskişehir - Türkiye
E-posta / E-mail : fsusuz@eskisehir.edu.tr

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Results: High doses of curcumin (30 µg/ml, 15 µg/ml, 7.5 µg/ml) showed high antiproliferative and cytotoxic effects on Hep2C cells. The antiproliferative and cytotoxic effects were not observed on cervical cancer Hep2C cells treated with lower concentrations of curcumin.

Conclusion: Curcumin has been shown that it is non-toxic, can be used as a highly antioxidant and anti-inflammatory agent and has multifaced therapeutic-pharmacological effects. However, researches on the antiproliferative, anti-cancer effects of curcumin in cervical cancer cells is not sufficient. The present study evaluates the antiproliferative and cytotoxic effects of curcumin on human cervical cancer Hep2C cells as the first time. The results of our study support these effects of curcumin on Hep2C cells in a concentration-dependent manner.

Key Words: Cancer, curcumin, MTT assay, cytotoxic effect, cell culture

Bulgular: Yüksek dozlarda kurkumin (30 µg/ml, 15 µg/ml, 7,5 µg/ml) Hep2C hücrelerinde yüksek oranda antiproliferatif ve sitotoksik etki göstermiştir. Daha düşük konsantrasyonda kurkumin uygulanan Hep2C servikal kanser hücre hattında antiproliferatif ve sitotoksik etki gözlenmemiştir.

Sonuç: Kurkuminin toksik olmadığı, yüksek oranda antioksidan ve antienflamatuvar ajan olarak kullanılabileceği ve çok yönlü terapötik- farmakolojik etkilere sahip olduğu gösterilmiştir. Ancak kurkuminin servikal kanser dokusundaki antiproliferatif, anti kanser etkileri konusundaki araştırmalar yeterli düzeyde değildir. Bu çalışmada kurkuminin ilk kez, servikal kanser hücresi Hep2C üzerindeki antiproliferatif ve sitotoksik etkisi mikroskobik yöntemlerle ve MTT testi aracılığı ile araştırılmıştır. Çalışmamızın sonuçları ile Hep2C hücrelerinde antiprolatif ve sitotoksik etkilerin konsantrasyona bağlı olarak artış gösterdiği ortaya konulmuştur.

Anahtar Kelimeler: Kanser, kurkumin, MTT testi, sitotoksik etki, hücre kültürü

INTRODUCTION

Cancer is a group of diseases caused by the accumulation of successive genetic and epigenetic alterations resulting in apoptosis resistance, uncontrolled cell proliferation, induced metastasis, angiogenesis, and dysfunction of the immune system (1, 2). Cancers arise by an evolutionary process as somatic cells mutate and escape the restraints that normally rein in their untoward expansion. Consequently, multiple mechanisms have arisen to forestall uncontrolled cell division. Some of these are devices within the cell, such as those that limit cell-cycle progression, whereas others are social signals that prompt a cell to remain within its supportive microenvironment (3).

Several strategies have been developed in recent years to combat cancer evasion, which includes surgery, chemotherapy, hormonal therapy and radiation therapy. The type of treatment to be used depends on the location, type, and progression of cancer as well as on the patient's health (4). Recent studies suggest the use of a few "adjuvants" in combination with chemotherapy to reduce the adverse effects and drug resistance and to increase the effective targeting of cancerous cells. Natural compounds, that are obtained from plants, are capable of garnering considerable attention from the

scientific community, primarily due to their ability to check and prevent the onset and progress of cancer (5).

Amongst the wide range of the medical herbs Curcumin is a yellow spice derived from the rhizomes of *Curcuma longa* commonly known as turmeric. It is a short-stemmed perennial that grows to about 100 cm in height (4). Curcumin belongs to a chemical class of polyphenols with a chemical formula of $C_{21}H_{20}O_6$ and a molecular weight of 368.38 (6). Curcumin has been used as a traditional medicine to treat a spectrum of diseases like rheumatism, body ache, skin diseases, intestinal worms, diarrhea, intermittent fevers, hepatic disorders, biliousness, inflammations, constipation, leukoderma, amenorrhea, arthritis, colitis and hepatitis (3, 7-9).

Many recent studies conducted by major research groups around world suggest the use of curcumin as a chemopreventive adjuvant molecule to maximize and the desired effects and minimize side effects of chemotherapeutic drugs. These studies have been shown that curcumin inhibits tumor growth by inhibiting cell cycle progression or by inducing apoptosis; by inhibiting angiogenesis, the expression of antiapoptotic proteins, multiple cell survival signaling pathways and their cross-communication; and by modulating immune responses (3, 10, 11). Several studies were conducted to explore the anti-cancer properties of curcumin and it was shown that curcumin modulates multiple cell signaling pathways which include cell proliferation, cell survival, apoptosis or cell death, as well as controls tumor suppressor pathway death receptor pathway, mitochondrial pathways, and protein kinase pathway, thereby affecting tumor cell growth (3, 10-12). Curcumin induces the initiation of both p53-dependent and p53-independent G2/M phase cell cycle arrest, thereby restricting cell proliferation and tumor progression. Therefore, curcumin is used either alone or in combination in targeting various types of cancers such as multiple myelomas, pancreatic, lung, breast, oral, prostate, and colorectal cancers, and head neck squamous cell carcinoma (4, 10-15).

In this study, antiproliferative and cytotoxic effects of different concentrations of commercial *Curcuma longa* extract (Neptun, Turkey) on cervical cancer Hep2C cell line were investigated with microscopic methods and MTT assay.

MATERIAL and METHOD

Materials

Curcuma longa extract commercial product purchased from Neptun, Turkey. Penicillin-streptomycin, dimethyl sulfoxide (DMSO), and MTT were obtained from Sigma-Aldrich (USA). Hep2C were used.

Cell culture

Hep2C cells were cultured in MEM (Minimum Essential Medium) (Gibco, USA) containing 10% FBS (Fetal Bovine Serum) (Gemini Byproducts, Calabasas, CA), 2 mM L-glutamine and 1% penicillin and streptomycin. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2. For continuity of the proliferated cells, the medium in the culture flasks was replaced with fresh MEM every third day. Confluent cells on the bottom of the culture flasks were washed with phosphate buffered saline (PBS) and harvested by treatment with trypsin solution (0.25% trypsin, 1 mM EDTA) for 3 min at 37 °C. For neutralizing the effect of trypsin, 2 mL of fresh culture medium was added before the mixture was centrifuged at 1200 rpm at room temperature. The pellets were resuspended with fresh MEM and reseeded in sterile culture flasks in a laminar flow cabinet. Subcultures were then incubated at 37 °C.

Nontreated cells were used as negative control and for positive control Hep2C cells were exposed to ammonium molibdate (1mg/ml) for the above given incubation period.

Cytotoxicity test (MTT assay)

For cytotoxicity evaluation, curcumin was dissolved in DMSO (14mM). Further dilutions were made with fresh MEM. Hep2C cells were harvested by

trypsinization and were plated at 10^5 cell/mL/ well into 96-well plates and were exposed to curcumin at concentrations of 30 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.7 µg/ml, 1.9 µg/ml, 0.9 µg/ml, 0.45 µg/ml for 24 h. After incubation at 37 °C in a 5% CO_2 humidified incubator, 20 µL/well of MTT solution (5 mg/mL in distilled water) was added and the plates were incubated for 3 h at 37 °C. The medium in each well was removed and changed with 100 µL of isopropanol. After mixing at room temperature, the plates were read with Spectramax I3 at 570 nm wavelength (n = 3). The graph of the absorbance data obtained by the PLA 2.0 application of Spectramax I3 device are presented in results. Each experimental and control group was repeated in triplicate. The percentage of viability was calculated by the following formula:

Cell proliferation (%) = [OD sample] × 100 / [OD control],

where OD is optical density.

Evaluation via inverted microscopy

Hep2C cells were plated onto sterilized coverslips in a petri dish and exposed to the different concentrations of curcumin, 30 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.7 µg/ml, 1.9 µg/ml, 0.9 µg/ml, 0.45 µg/ml, 0.25 µg/ml, 0.12 µg/ml for 24 h at 37 °C. After 24 h, the cells were washed in PBS. The preparations were observed based on changes in nuclei and structures using a inverted microscope (Leica Microsystems).

Statistical analysis

Statistical analyzes were performed by one-way analysis of variance for multiple comparisons of the GraphPad Prism 6.0. Significances of $p < 0.05$ level were taken in consideration.

RESULTS

Cytotoxicity of curcumin on Hep2C cells

Hep2C cells were exposed to curcumin at concentrations of 30 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.7 µg/ml, 1.9 µg/ml, 0.9 µg/ml, 0.45 µg/ml for 24 h. According to the cytotoxicity test results, curcumin inhibited cell viability in a concentration-dependent manner. At the concentrations of 30 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.7 µg/ml, the viability of Hep2C cells was decreased at statistically significant level ($p < 0.05$). The IC_{50} value of the agent for 24 h of exposure was detected as 4.1 µg/ml. Cell viability percentage decreased as the applied concentration increased. The sharpest decrease was detected at the highest concentration (Figure 1). Viability values of Hep2C cells calculated from the absorbances obtained from MTT assay are presented in Table 1.

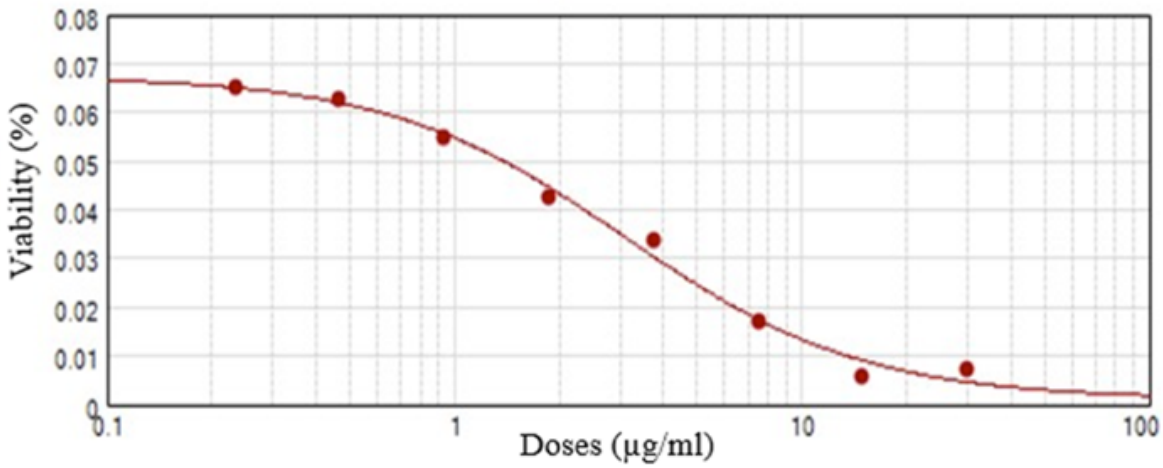

Figure 1. Antiproliferative activity of Curcumin on Hep2C cells for 24 hours.

Table 1. Viability values of Hep2C cells calculated from the absorbances obtained from MTT assay

Applied Curcumin Concentrations (µg/ml)	Hep2C IC_{50} Values
30	11.74
15	10.25
7.5	46.25
3.75	75.69
1.9	104.07
0.9	106.94
0.45	111.35

Effects of curcumin on Hep2C cell morphology using inverted microscopy

The morphological changes on Hep2C cells caused by 24 h of exposure to curcumin at concentrations of 30 µg/ml, 15 µg/ml, 7.5 µg/ml, 3.7 µg/ml, 1.9 µg/ml, 0.9 µg/ml, 0.45 µg, 0.25 µg/ml, 0.12 µg/ml are shown in Figures 2(A-I). Condensations of the nuclei and chromatin were the most significant morphological changes. Change in cell shape to circular was observed by inverted microscopy. In addition, a decrease in cell volume was recorded, whereas control cells remained unchanged (Figures 2J).

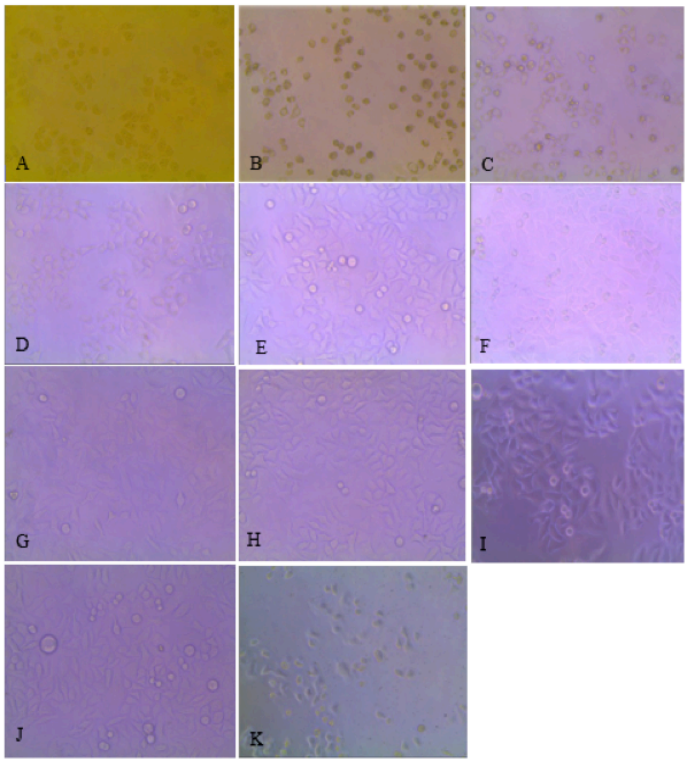

Figure 2. Microscope images showing the morphological changes of Hep2C cells exposed to curcumin at different concentrations (40X) for 24 hours.
(A) 30 µg/ml; (B) 15 µg/ml; (C) 7.5 µg/ml; (D) 3.7 µg/ml; (E) 1.9 µg/ml; (F) 0.9 µg/ml; (G) 0.45 µg/ml; (H) 0.25 µg/ml; (I) 0.12 µg/ml; (J) Negative control; (K) 1 mg/ml ammonium molibdate (positive control)

DISCUSSION and CONCLUSION

Recent studies suggest the use of few "adjuvants" in combination with chemotherapy to reduce the adverse effects and drug resistance and to increase the effective targeting of cancerous cells. Natural compounds, that are obtained from plants, are capable of garnering considerable attention from the scientific community, primarily due to their ability to check and prevent the onset and progress of cancer (5). Many recent studies conducted by major research groups around world suggest the use of curcumin as a chemopreventive adjuvant molecule to maximize the desired effects and minimize side effects of chemotherapeutic drugs (3, 10, 11).

The present study evaluates the antiproliferative and cytotoxic effects of curcumin on human cervical cancer Hep2C cells. The results of our study support the above effects of curcumin on Hep2C cells in a concentration-dependent manner. The statistically significant decrease of viability of test cells was determined in the highest four concentrations. As shown in the Table 1, the viability of the cells exposed to different curcumin concentrations for 24 h decreased remarkably in a dose-dependent manner. A significant decrease was recorded at the IC_{50} value (4.1 µg/ml) of curcumin for 24 h. At the level of the highest three concentrations, 30 µg/ml, 15 µg/ml, and 7.5 µg/ml, cell morphology deterioration and the dispersion of the cytoplasmic structure are clearly determined microscopically (Figure 2 A-C). Normal cell morphology is observed to be preserved at lower curcumin concentrations of 3.7 µg/ml, 1.9 µg/ml, 0.9 µg/ml, 0.45 µg/ml, 0.25 µg/ml, 0.12 µg/ml (Figure 2 D-I).

According to these data, it was determined that curcumin has an antiproliferative and cytotoxic effects on cervical cancer. Considering other studies investigating the anti-cancer activity of curcumin; Curcumin has been shown to reduce proliferation in the human leiomyosarcoma cells, human prostate and breast cancer cells (16-19).

In this study, the data obtained from the cervical cancer Hep2C cells, an anticancer herbal drug that can be produced from curcumin, it needs to be administered at high concentrations for curcumin to be effective in cancer therapy. According to the cervical cancer cells data in which curcumin is applied at lower concentration, it is seen that proliferation is not inhibited. In the light of these data, it has been shown "that curcumin has antiproliferative and cytotoxic effects. It is important for the production of a herbal anti-cancer drug candidate to study.

In conclusion, we recommend curcumin to be studied further as a potential agent for cancer treatment. Further in-depth investigations are required for other in vivo effects and elucidation of the mechanism of action.

ETHICS COMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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