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ÖZGÜR KORU

FERİT AVCU

MEHMET TANYÜKSEL

ALİ UĞUR URAL

REMZİ ENGİN ARAZ

See next page for additional authors

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Authors
ÖZGÜR KORU, FERİT AVCU, MEHMET TANYÜKSEL, ALİ UĞUR URAL, REMZİ ENGİN ARAZ, and KENAN ŞENER

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Özgür KORU[1]
Ferit AVCU[2]
Mehmet TANYÜKSEL[1]
Ali Uğur URAL[2]
Remzi Engin ARAZ[1]
Kenan ŞENER[3]

[1] Department of Microbiology, Division of Medical Parasitology, Gülhane Military Medical Academy School of Medicine, Ankara - TURKEY
[2] Department of Hematology, Gülhane Military Medical Academy School of Medicine, Ankara - TURKEY
[3] Department of Microbiology, Division of Virology, Gülhane Military Medical Academy School of Medicine, Ankara - TURKEY

Cytotoxic effects of caffeic acid phenethyl ester (CAPE) on the human multiple myeloma cell line*,**

Aim: Caffeic acid phenethyl ester (CAPE) has cytotoxic, apoptotic, and antiproliferative effects on various tumor cells, and is the most active component of propolis. This study aimed to examine the in vitro effects of CAPE on the human multiple myeloma cell line.

Materials and methods: CAPE was added to the ARH-77 multiple myeloma cell line and the percentage of dead cells was measured using the 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) assay. The percentage of live cells and growth inhibition were determined using the Trypan blue test. The percentage of IL-6 cells was determined using ELISA.

Results: ARH-77 cells treated with CAPE for 72 h at the 100 μg mL^{-1} concentration resulted in a growth inhibition effect of 90.4% and a cytotoxic effect of 80.4%. CAPE induced apoptosis in 92.3% of the cells in 22.5 μg mL^{-1} at 72 h. CAPE inhibited the secretion of IL-6 by ARH-77 multiple myeloma cells at LD_{50} concentrations.

Conclusion: CAPE inhibited growth and secretion of IL-6, and induced apoptosis in a dose-dependent and time-dependent manner in ARH-77 multiple myeloma cells. We think that CAPE merits further study as an effective agent against multiple myeloma.

Key words: Caffeic acid phenethyl ester (CAPE), multiple myeloma, cytotoxicity, IL-6

Kafeik asit fenetil esterin (CAPE) insan multiple myeloma hücre dizileri üzerinde sitotoksik etkileri

Genel bilgiler: Kafeik asit fenetil ester (CAPE), çeşitli tümör hücreleri üzerinde sitotoksik, apopitotik ve antiproliferatif etkilere sahip propolisin en aktif bileşiğidir. Bu çalışma da ilaç direnci ve nükslerin çok sık olarak görülmesine bağlı olarak tam bir tedavinin yapılamadığı insan multiple myeloma hücrelerinde CAPE'nin etkilerini inceledik.

Yöntem ve gereç: CAPE, ARH-77 multiple myeloma hücre dizileri üzerine konuldu ve 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) yöntemi ile ölen hücrelerin yüzdeleri ölçüldü. Yaşayan hücrelerin yüzdesi ve büyüme inhibisyonu, trypan blue testi ile tespit edildi. Hücrelerin IL-6 düzeyi yüzdesi ELISA ile hesaplandı.

Bulgular: ARH-77 hücrelerine 72. saat ve 100 μg mL^{-1} konsantrasyonunda uygulanan CAPE, % 90.4 büyüme inhibisyonu ve %80.4 sitotoksik etki oluşturdu. CAPE'nin, 72. saatte ve 22,5 μg mL^{-1} konsantrasyonunda hücrelerin % 92,3'ünde apoptozisi indüklediği, LD_{50} konsantrasyonunda ARH-77 hücreleri tarafından salınan IL-6 sekresyonunu inhibe ettiği bulundu.

Sonuç: CAPE'nin ARH-77 multiple myeloma hücreleri üzerinde doz ve zamana bağlı olarak büyüme, IL-6 sekresyonunu inhibe ettiğini ve apoptozisi indüklediğini bulduk. CAPE'nin multiple myelomaya karşı etkin bir ajan olarak ileriki çalışmalarda aday olabileceğini düşünmekteyiz.

Anahtar sözcükler: Kafeik asit fenetil ester, multiple myeloma, sitotoksitite, IL-6

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Correspondence
Özgür KORU
Department of Microbiology, Division of Medical Parasitology, Gülhane Military Medical Academy School of Medicine, Ankara - TURKEY

ozgurkoru@msn.com

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Introduction

Multiple myeloma (MM) is characterized by the presence of malignant plasma cells or plasma blasts localized predominantly in the bone marrow. Interleukin-6 (IL-6) is a growth factor for human myeloma cell lines. MM cannot be completely cured due to the high frequency of relapse and drug resistance. The uniformly fatal outcome of MM warrants development of novel biologically based treatment strategies that selectively induce apoptosis in malignant plasma cells (8,9). Recently, several agents, including curcumin, PS341, and thalidomide, have been tested in the search for a more effective treatment of MM. Nonspecific drug toxicity is one of the major problems in cancer drug treatment (10).

Propolis, known as bee glue, is a sticky substance that is dark-brown to yellow, depending on its origin. It is a resinous and waxy substance that bees collect from the buds and bark of trees (1). It has been reported that propolis has antibacterial and antifungal properties, and has been used by humans since early times for various purposes (1-3). Greenaway et al. identified 150 compounds in just 1 propolis sample (4); however, in total, more than 300 have been isolated to date. Progressive studies have shown that propolis has antimicrobial, anti-inflammatory, hepatoprotective, and anti-oxidative properties, and stimulates the immune system. Caffeic acid phenethyl ester (CAPE), which has anti-tumor, anti-inflammation, antiviral action, and antioxidant properties, is the most active component in propolis. CAPE has cytotoxic, apoptotic, and antiproliferative effects on various tumor cells, both in vivo and in vitro (5-7).

The present study aimed to investigate the ability of CAPE to induce apoptosis in the human MM cell line.

Materials and methods

Cell line

The immunoglobulin G-secreting plasma cell leukemia ARH-77 and λ light chain-secreting human MM cell line were generously supplied by the Karolinska Institute, Department of Hematology, Huddinge, Sweden. ARH-77 cells were maintained in RPMI 1640 medium supplemented with 10% and 20% heat-inactivated fetal calf serum, respectively, and 2 mM L-glutamine, 100 μg mL^{-1} of streptomycin, and 100 U mL^{-1} of penicillin at 37 °C in a 5% CO_2 atmosphere.

3-(4,5-Dimethyl-thiazoyl)-2,5-diphenyl-sh-tetrazolium bromide assay (MTT)

The MTT assay was performed as previously described (11). Target tumor cells (1×10^5 cells mL^{-1}) were resuspended in medium after verification of cell viability using the Trypan blue dye exclusion assay. Then 100 μL of cell suspension was distributed into each well of 96-well microtiter plates (Costar, Cambridge, MA, USA), and each plate was incubated for 24 h. Purified CAPE (≥99%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in DMSO (stock solution). Thereafter, 100 μL of reagent solution at the desired concentration (1, 5, 10, 25, 50, or 100 μg mL^{-1}) with a final DMSO concentration of 0.1% was distributed into each well and treated for 24, 48, and 72 h. Wells containing 200 μL of medium alone, without cells or reagents, were used as negative controls. Following treatment for the stated incubation times, 20 μL of MTT solution (5 mg mL^{-1}) was added to each well and the microplates were incubated for an additional 4 h at 37 °C. The unreactive supernatants in the well were discarded, and 100 μL of acidified isopropanol (0.04 N HCl in isopropanol) was added to the cultures and mixed thoroughly to dissolve the dark blue crystals of formazan. The absorbance value (A) of each well was determined with a microplate reader equipped with a 570-nm filter. The negative control well was used for baseline zero absorbance. Results are presented as the percentage of cytotoxicity, which was determined as follows: 1-A of experimental well/A of positive control well × 100. We calculated the dose of CAPE required to kill 50% (LD_{50}) of ARH-77 MM cells at 24, 48, and 72 h as a preliminary result for an animal study. Each experiment was repeated 3 times, with representative data presented.

DNA fragmentation assay

DNA fragmentation is one of the markers for induction of apoptosis (12). Cells were treated with different concentrations of CAPE for 24, 48, and 72 h. All the cells were harvested via centrifugation and

lysed with lysis buffer (5 mM Tris-HCl at pH 8.0, 20 mM EDTA, and 0.5% Triton X-100) on ice for 60 min. Cells were centrifuged at 14,000 rpm (45 min at 4 °C) and the supernatant fraction was transferred to a new tube. Then the quantity of protein was measured and the same amount of protein was used to normalize each sample for DNA fragmentation analysis. Fragmented DNA in supernatant fractions was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform, and precipitated with 100% ethanol and 5 M NaCl. The DNA pellet was washed once with 70% ethanol and resuspended in TE (10 mM Tris-HCl at pH 8.0, 1 mM EDTA at pH 8.0) buffer (pH 8.0) with 100 μg mL^{-1} of RNase A (Sigma) at 37 °C for 2 h. The DNA fragments were separated by 1.8% agarose gel electrophoresis and examined under ultraviolet light.

Cell growth kinetic

Cells were harvested after treatment with different concentrations of CAPE for 24, 48, and 72 h. The numbers of viable cells were counted using the Trypan blue dye exclusion test. Growth inhibition was calculated using the following equation: (cell number in the control group – cell number in treated group)/cell number in the control group. Each experiment was repeated 3 times, with representative data presented.

Assessment of apoptosis using an annexin-V FITC apoptosis kit

The LD_{50} concentration of CAPE caused a stimulating effect on apoptosis with the annexin V method via flow cytometry. The cells were treated and harvested as above. An annexin-V FITC apoptosis kit (Oncogene Research Products, Cambridge, MA, USA) was used to assess apoptosis, and then cells were immediately analyzed by flow cytometry. Early apoptotic cells were localized in the lower right quadrant of a dot-plot graph using annexin-V FITC. Each experiment was carried out on 3 different samples.

Detection of IL-6 by ELISA

The supernatants of the cells with and without CAPE in LD_{50} concentration were collected and the IL-6 level of these cells was detected by ELISA (BioSource International); each experiment was repeated 3 times.

Statistical analysis

Results are expressed as mean ± standard error of at least 3 experiments. Statistical comparisons were based on Kruskal-Wallis regression analysis or analysis of variance. Differences were considered significant at $P < 0.05$. All statistical analyses were carried out with SPSS v.12.0 software (SPSS, Inc.).

Results

The cytotoxic effect of various concentrations of CAPE (1-100 μg mL^{-1}) on the ARH-77 MM cell line with MTT assay is shown in Table 1. There was not a significant difference in the percentage of dead cells in the 1 or 5 μg mL^{-1} concentrations at 24, 48, or 72 h, whereas in the 10, 25, 50, and 100 μg mL^{-1} concentrations of CAPE at 24, 48, and 72 h the statistical difference in the percentage of dead cells was significant. The cytotoxic effect of CAPE was observed on ARH-77 cells, depending on the concentration and duration of application (Figure 1).

There was not a significant difference in the percentage of viable cells in the 1, 5, 10, 50, or 100 μg mL^{-1} concentrations of CAPE at 24, 48, or 72 h, whereas the decrease in viable cells in the 25 μg mL^{-1} CAPE concentration at 24, 48, and 72 h was statistically significant ($P = 0.002$) (Table 2). Also, a significant statistical difference was observed in the 25 μg mL^{-1} CAPE concentration between hours (24-48 h: $P = 0.045$; 24-72 h: $P = 0.001$; 48-72 h: $P = 0.007$). At 72 h it was obvious that the number of viable cells decreased as the CAPE concentration increased (Figure 2).

Table 1. MTT assay-based evaluation of the cytotoxic effect of CAPE on ARH-77 MM cells at various concentrations applied for 24, 48, and 72 h.

Concentrations (μg mL^{-1})	24 h (%)	48 h (%)	72 h (%)	P
1	1.1	10.1	15.0	0.109
5	3.0	27.2	18.6	0.117
10	3.5	29.7	39.0	0.015
25	9.3	44.3	58.7	0.001
50	63.0	77.4	78.3	0.001
100	65.7	79.4	80.4	0.001

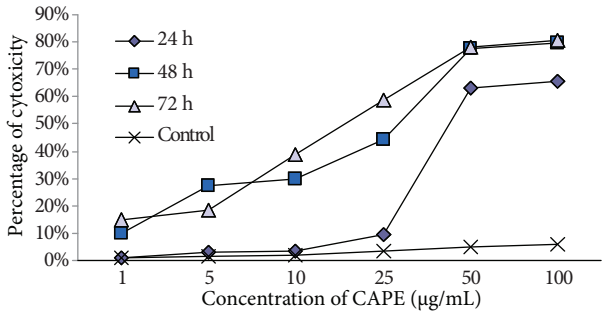

Figure 1. A graph of the percentage cytoxicity of CAPE on the ARH-77 cells in various concentrations at 24th, 48th and 72nd hour. The data depict the mean ± SD of 3 separate experiments.

Table 2. Evaluation of the percentage of viable ARH-77 MM cell cultures after the application of CAPE at different concentrations for 24, 48, and 72 h, based on the Trypan blue viability test.

Concentrations (µg mL^{-1})	24 h (%)	48 h (%)	72 h (%)	P
1	94.5	91.1	91.6	0.480
5	87.0	88.7	72.5	0.053
10	85.2	83.7	64.3	0.118
25	69.3	49.1	26.5	0.002
50	30.8	13.7	9.8	0.293
100	17.5	4.3	3.7	0.098

CAPE was applied to ARH-77 human MM cells (1, 5, 10, 25, 50. and 100 µg mL^{-1} concentrations) and the percentage of dead cells was determined after 24, 48, and 72 h. The LD_{50} values of CAPE at 24, 48, and 72 h are shown in Table 3. With increased duration of treatment the LD_{50} decreased.

Microscopic analysis of cell cultures to which CAPE was applied for 24, 48, and 72 h showed apoptotic changes, such as membrane blebbing and cell fragmentation into apoptotic bodies (Figure 3). At later times few surviving cells were observed.

The effect of treating cells with a CAPE LD_{50} concentration was measured using anexin V and flow cytometry (Figure 4). CAPE induced apoptosis in 57.7% of the myeloma cells at the LD_{50} concentration of 49.1 µg mL^{-1} at 24 h, 84.9% of the cells at 30.6 µg mL^{-1} at 48 h, and 92.3% of the cells in 22.5 µg mL^{-1} at 72 h (Table 4).

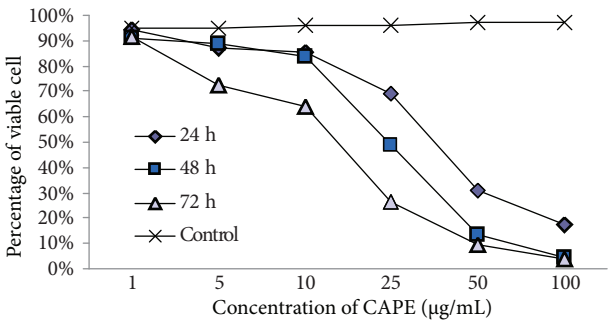

Figure 2. The effect of increased CAPE concentrations on the viability of ARH-77 human MM cell cultures is shown.

Table 3. The LD_{50} of CAPE at 24, 48, and 72 h.

Time	LD_{50} values	R
24	49.1	.874
48	30.6	.895
72	22.5	.827

The supernatants of the cells with and without an LD_{50} concentration of CAPE were collected and the concentration of IL-6 was detected by ELISA. At 24 h untreated cells expressed 15.9 pg mL^{-1} of IL-6, whereas treatment with CAPE lowered this level to 13.6 pg mL^{-1} at the LD_{50} concentration of 49.1 µg mL^{-1}. Likewise, CAPE treatment lowered IL-6 secretion from 38 pg mL^{-1} to 31.2 pg mL^{-1} at 48 h and from 73.5 pg mL^{-1} to 15.6 pg mL^{-1} at 72 h (Table 4). As a result, LD_{50} concentrations of CAPE inhibited secretion of IL-6 by ARH-77 MM cells at the following percentages: 12.9% at 24 h, 17.9% at 48 h, and 78.8% at 72 h (Figure 5).

DNA gel electrophoresis, which shows DNA fragmentation, is a very effective way to distinguish apoptosis from necrosis. This method is used in almost every study to evaluate the apoptotic effect of CAPE. In order to detect the effect of CAPE on myeloma cells, DNA of the cells treated with/without CAPE at LD_{50} concentration was subjected to 1.8% agarose gel electrophoreses. The DNA of the cells exposed to CAPE was fragmented at about 180-200 bp, which is consistent with cells undergoing apoptosis (Figure 6).

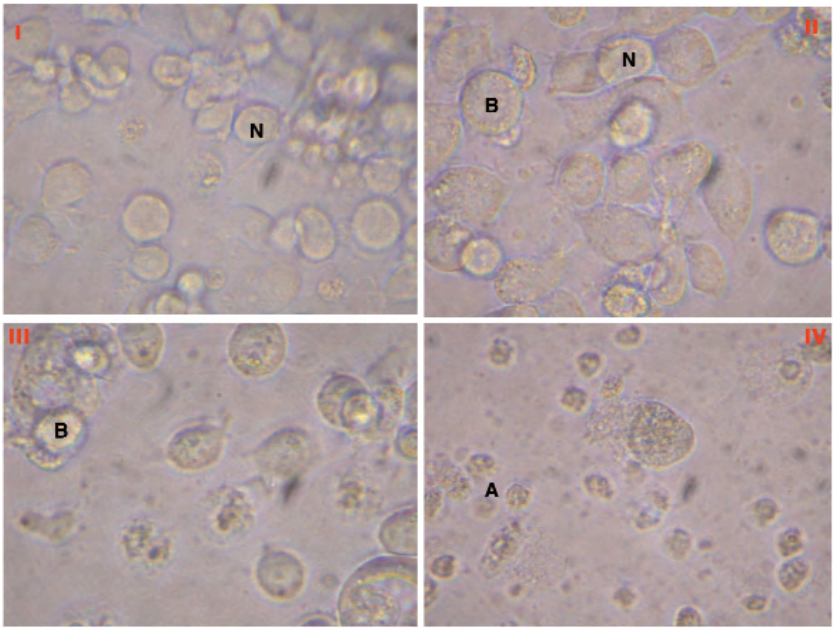

Figure 3. The microscopic views of MM cell culture with and without CAPE at concentrations at the 24th, 48th and 72nd hour (40X). (I. Control, II. 24th hour, III. 48th hour, IV. 72nd hour; N: Normal MM cell, A: Apoptotic MM cell, B: Apoptotic Bodies).

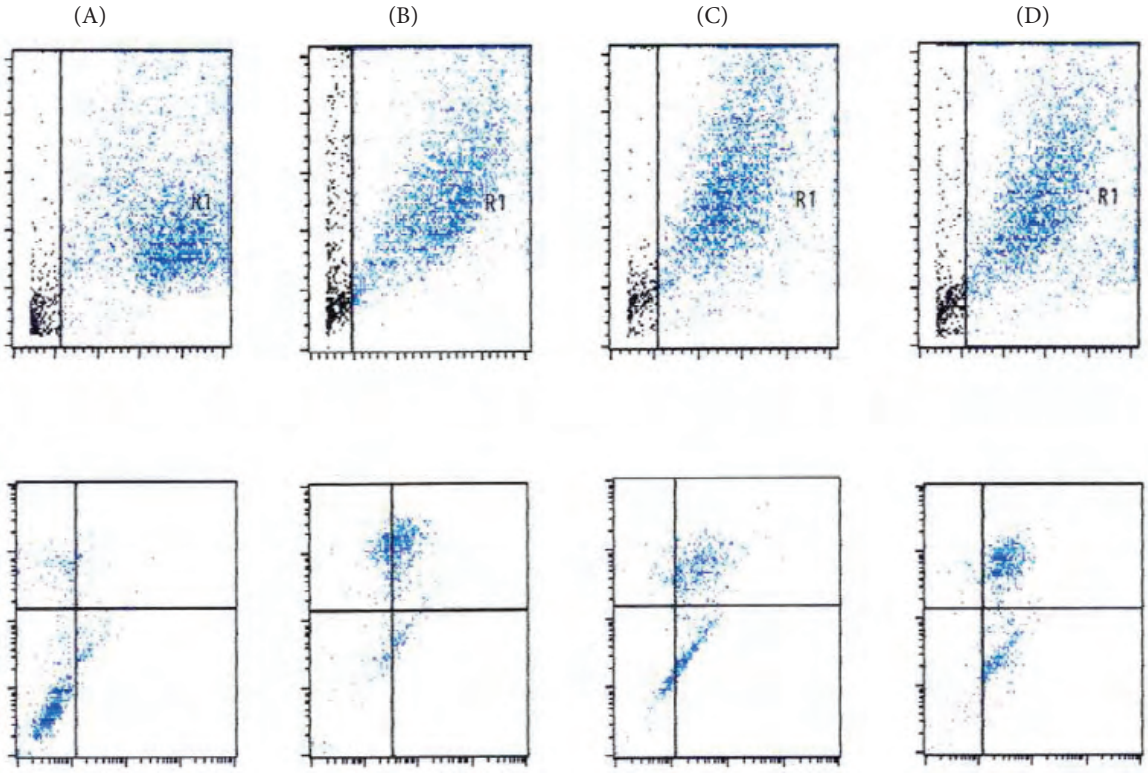

Figure 4. Measurement of the apoptosis stimulating effect of CAPE in LD_{50} concentrations at the 24th, 48th and 72nd hour with Annexin V method by flow cytometry (A: Control, B: 24 hours with 49.1 µg/mL, C: 48 hours with 30.6 µg/mL, D: 72 hours 22.5 µg/mL).

Table 4. Evaluation of the inhibition of IL-6 secretion by ARH-77 MM cells due to CAPE at LD_{50} concentrations at 24, 48, and 72 h.

Time	LD_{50} values	Control cell IL-6 (pg mL^{-1})	With CAPE cell IL-6 (pg mL^{-1})	IL-6 inhibition (%)
24	49.1	15.6	13.6	12.9
48	30.6	38.0	31.2	17.9
72	22.5	73.5	15.6	78.8

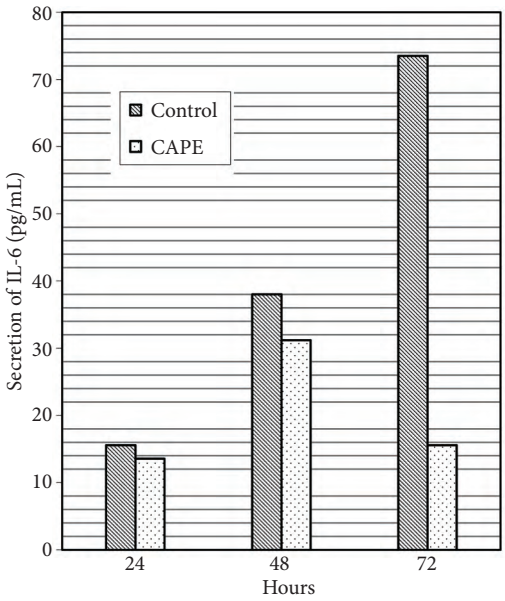

Figure 5. Evaluation of inhibition secretion of IL-6 by ARH-77 multiple myeloma cells, at LD_{50} concentrations according to hours.

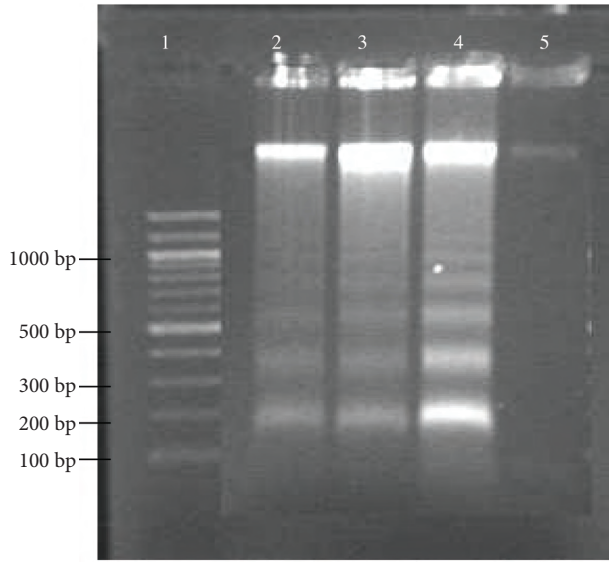

Figure 6. Induction of DNA fragmentation in ARH-77 MM cells by CAPE in the LD_{50} concentrations at 24^{th}, 48^{th} and 72^{nd} hour (Lane 1, 100-bp DNA ladder, lane 2, 24 h, lane 3, 48 h, lane 4, 72 h, lane 5, untreated cells).

Discussion

Recent studies show that CAPE has a strong cytotoxic effect on various tumor cell lines and that it has anti-oxidative properties (5-7,13-25). There have not been any studies on the cytotoxic effect of CAPE on MM cells, which are resistant to treatment. Inhibition of apoptosis is a very important mechanism of tumor formation and can cause resistance to chemotherapeutic drugs. Many chemotherapeutic agents may act via the induction of apoptosis to block the carcinogenic process. It is emphasized that the anti-tumor activity of CAPE results from the induction of apoptosis (14). When compared to other chemotherapeutic agents, an advantage of CAPE is that it is 100% natural, as it is a component of propolis, which is collected by bees from the bark and buds of trees. Studies show that propolis has no side effects, except for slight allergic reactions, and is not toxic to normal cells (26).

The cytotoxity rates and LD_{50} values we observed were much higher than previously reported for different cancer cells. Only the cytotoxity rate on prostate cancer cells reported by McEleny et al. (22) was similar to the cytotoxity rate of CAPE at 24 h in the present study. We think that this similarity is a result of the high resistance of both PC-3 prostate cancer cells and ARH-77 cells to cytotoxity and apoptosis.

CAPE not only inhibits tumoral cell growth due to its antioxidant effect, but also induces apoptosis in tumoral cells due to its cytotoxic properties. Chen et al. (6) reported that CAPE induced growth inhibition

in 70.3% and induced apoptosis in 41.7% of human leukemic HL-60 cells via selective scavenging of hydrogen peroxide, mitochondrial dysfunction, and intracellular glutathione (GSH) depletion. We also detected that the growth inhibiting effect of CAPE was stronger than its cytotoxic effect.

Chen et al. (13) reported such results in a study they performed on A549 human lung adenocarcinoma cells. In many other studies, as in ours, the annexin V method by flow cytometry was used to test the apoptosis-stimulating effect of CAPE on cancer cells (6,13,22-25). Most of the apoptosis rates in various tumor cells were similar.

Dysregulation of the apoptotic mechanism in plasma cells is considered a major underlying factor in the pathogenesis and subsequent chemoresistance in MM. IL-6 plays an important role in the progression of MM malignancy by regulating the growth and survival of tumor cells. Studies have shown that secretion of IL-6 leads to expression of high levels of anti-apoptotic proteins, such as protein B-cell lymphoma-x_L (Bcl-x_L) and that over-expression of this protein is a very important characteristic of MM cell lines. Additionally, in MM cells IL-6 functions as an osteoclast activator. It has been shown that the level of IL-6 is related to disease activity. IL-6 is produced in either an autocrine or paracrine manner (10,27). As mentioned above, we observed that CAPE inhibited secretion of IL-6 by ARH-77 MM cells at LD_{50} concentrations, with the following percentages: 12.9% at 24 h, 17.9% at 48 h, and 78.8% at 72 h. Some researchers reported that there must be interaction between bone marrow stromal cells and malignant plasma cells for the production of IL-6 (27,28); however, Alsina et al. (9) showed that there was no IL-6 in the sera or bone marrow plasma cells of MM-infected mice, but that ARH-77 MM cells secreted 120 mg mL^{-1} of IL-6 in vitro. In the present study we noted that ARH-77 MM cells secreted 15.6 pg mL^{-1} of IL-6 at 24 h, 38 pg mL^{-1} of IL-6 at 48 h, and 73.5 pg mL^{-1} of IL-6 at 72 h.

Consequently, we think that CAPE could be used to treat MM in the future, because of its ability to inhibit human ARH-77 MM cell growth and induce apoptosis. Further research is needed to confirm this hypothesis.

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