RESEARCH COMMUNICATION

Apoptosis-Mediated Inhibition of Human Breast Cancer Cell Proliferation by Lemon Citrus Extract

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Abstract

Dietary phytochemicals have a variety of antitumor properties. In the present study, the antitumor activity of methanolic extract of lemon fruit (lemon extract; LE) (LE) on the MCF-7 breast cancer cell line was investigated in vitro. Apoptotic cell death was analyzed using the TUNEL assay. In addition, the apoptosis mediated by LE extract in the MCF-7 cells was associated with the increased expression of the tumor suppressor p53 and caspase-3. Additionally, the expression of a pro-apoptotic gene, bax, was increased, and the expression of an anti-apoptotic gene, bcl-2, was decreased by LE extract treatment, resulting in a shift in the Bax:Bcl-2 ratio to one that favored apoptosis. The expression of a major apoptotic gene, caspase-3, was increased by LE extract treatment. In light of the above results, we concluded that LE extract can induce the apoptosis of MCF-7 breast cancer cells via Bax-related caspase-3 activation. This study provides experimental data that are relevant to the possible future clinical use of LE to treat breast cancer.

Keywords: Lemon citrus - apoptosis - MCF-7 cells - gene expression - mitochondrial membrane potential

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Introduction

The development of new strategies for the treatment of cancer is urgently needed. The mechanism of action of many anticancer compounds or drugs is based on their ability to promote apoptosis. There are many mechanisms by which apoptosis can be induced in cells (Sen et al., 1992; Motomura et al., 2008). Compounds suppressing the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy. However, the unfavorable side effects and the development of resistance to many currently used anticancer agents are serious problems (Khan et al., 1999). Furthermore, the use of plant materials and extracts as alternative cancer therapies has attracted much recent attention due to the low toxicity and low cost of plant-derived materials (Zhang et al., 2005).

Natural compounds in food with diverse bioactivities are becoming an important source of novel agents with pharmaceutical potential, and these compounds have attracted a great deal of attention in recent years because of their role in modulating the development of certain types of human cancers (Chen et al., 2007). The ingestion of fruits and vegetables was shown to have a significant protective effect (Sen et al., 1992), and the risk of cancer is higher in persons with a low intake of fruits and vegetables than in those with a high intake. Citrus fruit, which is commonly used in traditional medicine in China and other countries (Luo et al., 2008), has an antiproliferative effect on many types of cancers, including but not limited to breast cancer. Therefore, the present study was carried out to determine if LE extract is able to induce apoptosis in MCF-7 cells and to determine the mechanism responsible for its activity.

Materials and Methods

Preparation of plant extract

Plant material was pulverized using a milling machine and extracted with methanol using a Soxhlet apparatus as described previously [Shafi et al 2009]. The organic phase was evaporated under reduced pressure to obtain a residue. The residue was dried using a rotary evaporator to obtain the powder/paste. The required quantity of the dry powder/paste was dissolved in dimethyl sulfoxide (DMSO).

Maintenance of MCF-7 cells

The MCF-7 breast cancer cell line was a kind gift from Dr. M. A. Akbarshah at the Mahatma Gandhi-Doerenkamp Center (MGDC) for Alternatives to Use of Animals in Life Science Education, Bharathidasan University,

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India. The cell line was maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were cultured as adherent monolayers (i.e., cultured at approximately 70% to 80% confluence) and maintained at 37°C in a humidified atmosphere of 5% CO_2. Cells were harvested after brief trypsinization). All chemicals used were of research grade.

Toxicity assay (MTT assay)

Cells were grown in DMEM at 37˚C under 5% CO2 in a humidified incubator. Cells were harvested, counted and transferred to 96-well plates and incubated for 24 h prior to the addition of the test compounds. The extracted compounds were processed and applied in various concentrations, and the treated cells were incubated for 48 h. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (5 mg) was dissolved in 1 ml of phosphate-buffered saline (PBS), and 25 μl of the MTT solution was added to each of the 96 wells. The plates were wrapped in aluminum foil and incubated at 37˚C for 3 h. The solution in each well, containing media, unbound MTT and dead cells, was removed by suction, and 200μL of DMSO was added to each well. The plates were then shaken, and the optical density was measured using a microplate reader at 575nm.

Caspase-3 activity assay

A commercially available kit (Promega) was used to measure the activity of caspase-3 according to the manufacturer's protocol. After treatment with LE, MCF-7 cells were lysed in lysis buffer (25, 50, 100, or 200 μg/mL) for 24 h. Active caspase-3 was used as a positive control. All mixtures were incubated overnight in a humidified environment at 37°C. The amount of p-nitroaniline (p-NA) released from the substrate was measured.

TUNEL assay

The DeadEnd® TUNEL assay kit (Promega, Madison, WI) was used to analyze apoptosis in a time-dependent manner. The manufacturer's instructions were followed with slight modifications. Briefly, MCF-7 cells (1.5 x 106 cells/well) were cultured in 6-well plates to study apoptosis in adherent cells. The cells were treated with 50 μg/mL or 100 μg/mL LE extract for either 24 or 48 h. After the incubation period, the culture medium was aspirated, and the cell layers were trypsinized. The trypsinized cells were reattached on 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and stained according to the DeadEnd fluorometric TUNEL system protocol (Wang et al., 2004). The stained cells were observed using a Carl-Zeiss (Axiovert) epifluorescence microscope using a triple band-pass filter. To determine the percentage of cells undergoing apoptosis, 1000 cells were counted in each experiment [Shafi et al 2009].

Real-time quantitative PCR analysis

The expression of apoptotic genes was analyzed by reverse transcription-PCR (RT-PCR; Applied Biosystems, Foster City, CA) using a real-time SYBR Green/ROX gene expression assay kit (Qiagen). The cDNA was directly prepared from cultured cells using a FastLane® Cell cDNA kit (Qiagen, Germany), and the mRNA levels of caspase-3, bax, TNF alpha, bcl-2, mdm2 and TP53 as well as the reference gene, gapdh, were assayed using gene-specific SYBR Green-based QuantiTect® Primer assays (Qiagen, Germany). Quantitative real-time RT-PCR was performed in a reaction volume of 25 μL according to the manufacturer's instructions. Briefly, 12.5 μL of master mix, 2.5 μL of primer (10x) and 10 μL of template cDNA (100 μg) were added to each well. After a brief centrifugation, the PCR plate was subjected to 35 cycles using the following conditions: (i) PCR activation at 95°C for 5 min; (ii) denaturation at 95°C for 5 s; and (iii) annealing/extension at 60°C for 10 s. All samples and controls were run in triplicate on an ABI 7500 Fast Real-time PCR system. The quantitative RT-PCR data were analyzed using the comparative threshold (Ct) method, and the fold inductions of the samples were compared with those of the untreated samples. GAPDH was used as an internal reference gene to normalize the expression of the apoptotic genes. The Ct cycle was used to determine the expression level in control cells and MCF-7 cells treated with LE for 24 and 48 h. The gene expression level was then calculated as described by Yuan et al. (2006). The results were expressed as the ratio of reference gene mRNA to target gene mRNA using the following formula: ΔCt = Ct (apoptotic genes) – Ct (GAPDH). To determine the relative expression levels, the following formula was used: $\Delta\Delta$Ct = ΔCt (Treated) - ΔCt (Control). Thus, the expression levels were expressed as n-fold differences relative to the expression of the reference gene. The values were used to plot the expression of apoptotic genes using the expression 2-$\Delta\Delta$Ct.

Mitochondrial membrane potential ($\Delta\psi$m) analysis

$\Delta\psi$m was analyzed using JC-1 (Promega). JC-1, a fluorescent compound, exists as a monomer at low concentrations and form aggregates at higher concentrations. The fluorescence of the JC-1 monomer is green, whereas that of the aggregate is red. Mitochondria with intact membrane potentials concentrate JC-1 into aggregates; hence, the mitochondria fluoresce red. De-energized mitochondria cannot concentrate JC-1 and fluoresce green (Keshavan et al., 2004). Cells were exposed to LE extract for 24 h at various concentrations. Control cells were grown in medium alone. Then, the cells were incubated in 0.5 ml of medium containing JC-1 (2.5 µg/ml) for 30 min at 37°C, and images were taken using a Carl-Zeiss epifluorescence microscope with a triple band-pass filter.

Results

Cytotoxicity assay

Methanol was used to extract the anticancer components from LE. The effect of various concentrations of the methanolic extract of LE on the growth of MCF-7 cells was subsequently examined using an MTT-based assay. The percent viabilities of MCF-7 cells exposed to the methanolic extract of LE at 25, 50, 100, or 200μg/mL were 68.3% ± 1.9%, 27.2% ± 4.8%, 15.1% ± 2.2%, and

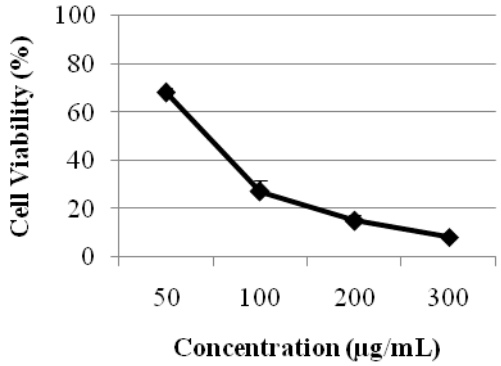

Figure 1. Effect of the Methanolic Extract of LE on MCF-7 Cell Viability. Cells were treated with the extract of lemon fruit. Untreated (DMSO alone) cells were used as controls. Viability was determined using MTT reduction. The values presented are the means ± SD

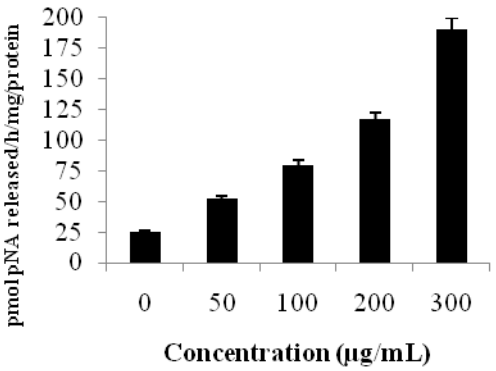

Figure 2. Effect of the Methanolic Extract of LE on Caspase-3 Activity MCF-7 Cells. Cell lysates prepared after treatment with the LE extract for 24 h. The rate of cleavage of the caspase substrate DEVD-p-NA was measured at 405 nm. The results are mean ± SD of triplicate experiments

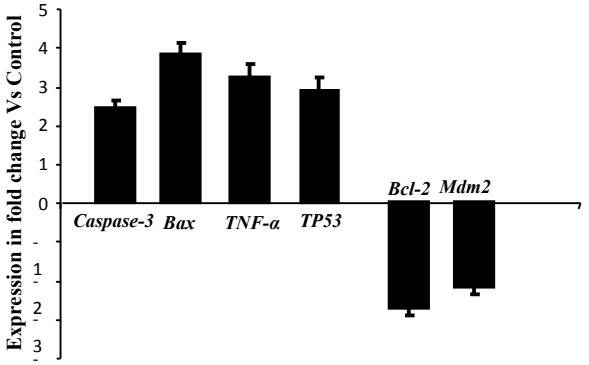

Figure 3. Comparison of the Change in Expression of Apoptosis-related Genes. Data are expressed as the fold change (ratio of target:reference gene) in MCF-7 cells after 24 h of exposure to 50 µL/mL of the methanolic extract of LE. The values presented are the means ± SD

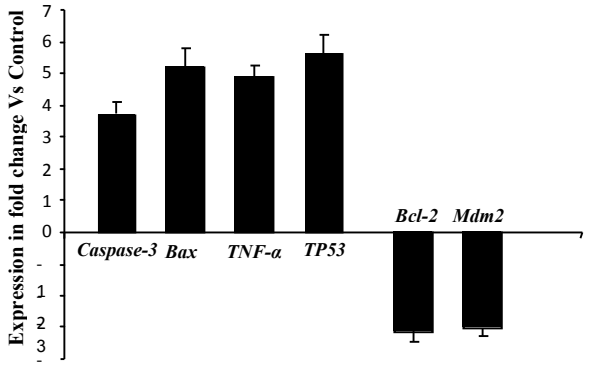

Figure 4. Comparison of the Change in Expression of Apoptosis-related Genes. Data are expressed as the fold change (ratio of target:reference gene) in MCF-7 cells after 24 h of exposure to 100 µL/mL of the methanolic extract of LE. The values presented are the means ± SD

8.4% ± 0.5%, respectively, compared with the controls ($p < 0.001$); moreover, the inhibition of cell growth was dose-dependent (Figure 1).

Caspase-3 activity

We also examined caspase activation in the LE extract-treated cells. LE -extract treatment increased caspase-3 activity in a dose-dependent manner, as shown in Figure 2. These results suggest that the apoptotic effects of LE extract on MCF-7 cells are associated with an increase in caspase activation.

Quantification of apoptosis using a TUNEL assay

To determine whether the inhibition of cell proliferation by LE extract was due to the induction of apoptosis, a TUNEL assay was used. A dose-dependent increase in the induction of apoptosis was observed when MCF-7 cells were treated with LE extract. When compared to the control cells at 24 h, after normalization to the control, 10.7% and 29.2% of the cells treated with 50µg/mL or 100µg/mL LE extract, respectively, underwent apoptosis.

Quantification of mRNA levels of apoptosis-related genes

To investigate the molecular mechanism of LE extract-induced apoptosis in MCF-7 cells, the expression levels of several apoptosis-related genes were examined. Bcl-2, Bax and p53 are three major proteins generally involved in apoptosis. It is unknown if LE extract induces or inhibits the expression of these genes. The relative quantification of caspase-3, bax, TNF alpha, bcl-2, mdm2 and TP53 mRNA levels was performed using SYBR Green-based quantitative real-time reverse transcription PCR (RT-PCR) using a 7500 Fast Real-Time System (Applied Biosystems).

Figures 4 and 5 summarize the gene expression changes of caspase-3, bax, TNF alpha, bcl-2, mdm2 and TP53. LE extract treatment increased the number of transcripts of caspase-3, bax, TNF alpha, and TP53 by several fold. The expression levels of these genes in MCF-7 cells treated with 50 or 100µg/ml LE extract for 24 h increased by 2.41-, 3.83-, 3.25-, and 2.9-fold, respectively, as compared to the levels in untreated control cells. In a time-dependent manner, the expression levels of the above genes in MCF-7 cells treated with 50 or 100µg/ml LE extract for 48 h increased when compared to the levels in untreated control cells. The expression of bcl-2 was inhibited in a time-dependent manner, whereas bax expression was increased in LE extract-treated cells, indicating that the LE -extract treatment induced apoptosis by shifting the Bax:Bcl-2 ratio. Moreover, the Mdm2:p53 ratio was higher in LE extract-treated cells.

Mitochondrial membrane potential

A cellular functional assay was performed to estimate

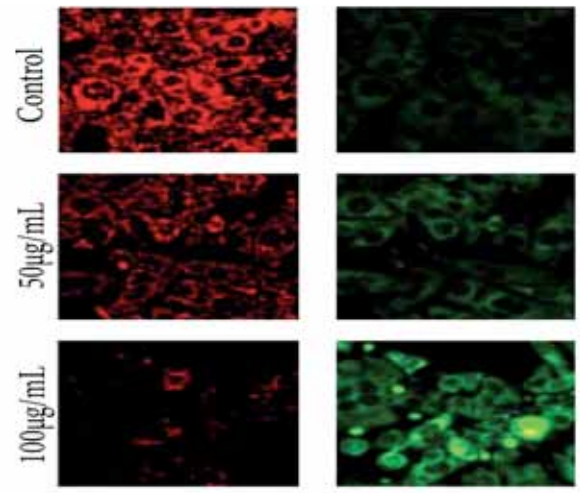

Figure 5. Analysis of Mitochondrial Membrane Potential after LE Extract Exposure. MCF-7 cells were exposed to the methanolic extract of LE for 24 h and then stained with 2.5 μM of JC-1 for 20 min. Red fluorescence indicates mitochondria with intact membrane potentials. Green fluorescence indicates de-energized mitochondria. Images were taken with a Carl-Zeiss epifluorescence microscope

the spatial variation in mitochondrial membrane potential ($\Delta\psi$m) using JC-1. This compound accumulates in the mitochondria as the result of the membrane potential. At low $\Delta\psi$m, mitochondria accumulate fewer JC-1 molecules and fluoresce green (485 excitation/535 emission), whereas at high concentrations (high $\Delta\psi$m), the compound aggregates and exhibits a red fluorescence (535 excitation/590 emission). The loss of mitochondrial membrane potential is followed by a red-to-green shift. After treatment with two different concentrations of the extract (50 and 100μg/ml), $\Delta\psi$m decreased. After treatment with the extract (50 and 100μg/ml), the fluorescence shifted from red to green, as shown in Figure 5, indicating the loss of mitochondrial function. This result suggests that the extract induced apoptosis via the mitochondrial pathway

Discussion

Several natural compounds with anticancer effects can induce apoptosis of tumor cells (Tsuda et al., 2004; Shafi et al., 2009). LE extract also induced apoptosis in MCF-7 cells. To understand the potential antitumor mechanisms of the LE extract, the relative expression levels of the Bcl-2, Bax, TNF alpha, Mdm2 and p53 genes induced by lemon citrus were detected by RT-PCR. We found that Bcl-2 and Mdm2 expression was decreased, whereas the expression of Bax, TNF alpha and p53 was increased; therefore, the Bax:Bcl-2 ratio was elevated.

LE extract may induce apoptosis through p53, through the downregulation of Bcl-2 and Mdm2 and through the upregulation of Bax. Apoptosis occurs through the extrinsic (cytoplasmic) pathway and/or the intrinsic (mitochondrial) pathway (Guessous et al., 2007). The Bcl-2 family is one of the most important classes of regulators involved in the intrinsic pathway. The functions of Bax and Bcl-2 are known to be upstream of caspases that regulate apoptosis promoted by different stimuli (Gao et al., 2005; Chang et al., 2006).

The possible mechanism by which p53 regulates apoptosis involves activating the mitochondria-regulated death pathway by elevating gene expression of pro-apoptosis genes in the Bcl-2 family and suppressing the expression of anti-apoptotic genes (Oren et al., 2003; Green et al., 2004). The p53 protein interacts with Bcl-2 to enhanced Bax-promoted outer mitochondrial membrane permeabilization, and p53 is a direct transcriptional activator of the Bax gene (Miyashita et al., 1995; Butt et al., 2000).

Increased expression of p53 induces an increase in the Bax:Bcl-2 ratio, resulting in the release of cytochrome c, caspase activation, and, ultimately, apoptosis (Luo et al., 2008). One possible mechanism by which Bax may function in the p53-mediated cell death pathway is through the activation of caspases: p53-mediated activation of caspase-3 is dependent on Bax (Cregan et al 1999]. Bax is required for caspase activation after potassium withdrawal-enhanced cell death of cerebellar granule neurons (Miller et al., 1997). Furthermore, Bax overexpression can promote caspase activation in neuronal cells (Vekrellis et al., 1997;Martinou et al., 1998). Another major physiological regulator of p53 function is Mdm2. Mdm2 specifically binds to p53 and inhibits its biochemical activities. Furthermore, through its action as a p53-specific E3 ubiquitin ligase, Mdm2 ubiquitinates p53 and targets it for rapid proteasomal degradation (Oren, 1999; Momand et al., 2000). Our data suggest that LE extract may induce cell apoptosis mediated by p53 through the downregulation of Mdm2.

Apoptosis is also the result of death receptor-dependent (extrinsic) or death receptor-independent (intrinsic or mitochondrial) mechanisms (Fulda and Debatin 2006]. The best-characterized death receptors belong to the tumor necrosis factor (TNF) superfamily. The corresponding ligands of the TNF superfamily are the death receptor ligands.

By inducing the release of mitochondrial cytochrome c, p53 might be able to activate effector caspases including caspase-3. Taken together, these activities suggest a potential mechanism for the induction of apoptosis by lemon extract. LE extract may induce apoptosis mediated by p53 through the downregulation of Bcl-2 and the upregulation of Bax. LE extract showed antitumor effects on breast cancer cells, and these results suggest that lemon citrus can induce p53-mediated apoptosis via modulation of the Bax:Bcl-2 ratio. Protein expression data are needed to confirm the changes observed at the mRNA level.

In conclusion, the present study demonstrated that the LE extract inhibited cancer cell proliferation via the induction of apoptosis. LE extract-induced MCF-7 cell death was shown to be due to apoptosis as demonstrated by the induction of caspase-3 activity and the observation of cells containing fragmented nuclei and DNA. This apoptotic response is associated with the upregulation of Bax, the downregulation of Bcl-2 and caspase activation. Therefore, we suggest that LE extract is a promising molecule in cancer chemoprevention or chemotherapy. These results indicate that the LE fruit extract has anticancer activity in vitro. Further studies are

warranted to determine the molecular mechanisms of the active components and to evaluate the potential in vivo anticancer activity of the extract.

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