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Levisticum Officinale Extract Triggers Apoptosis and Down-Regulates *ZNF703* Gene Expression in Breast Cancer Cell Lines

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Abstract

Background: Studies have shown that zinc finger protein 703 (ZNF703) is overexpressed in breast cancer. *Levisticum (L.) officinale* is a herbal plant with proven medical characteristics in traditional medicine. The purpose of the present study was to evaluate the effect of hydroalcoholic extract of *L. officinale* (HELO) on both estrogen receptor-positive (ER^+) and -negative (ER^-) cell lines (MCF-7 and MDA-MB-468, respectively).

Methods: The anti-proliferative and apoptotic activities of HELO were investigated on both cell lines using MTT and flow-cytometry methods. Real-time PCR was employed to determinate the changes in mRNA expression of the *ZNF703* gene.

Results: The 50% maximal inhibitory concentrations (IC_{50s}) of HELO on ER^+ and ER^- cells were 200 and 150 µg/mL after 48 h-treatment. Statistically significant increases in both early and late apoptosis rates were seen in exposed cell lines. *ZNF703* expression was less from 4 to 24 h HELO treatment than in untreated cells, and *ZNF703* expression was higher in the more invasive MDA-MB-468 cells than in the less invasive MCF-7 cells. Our results demonstrated that HELO induces apoptosis and decreases cell growth in both cell lines.

Conclusions: Our data suggest that HELO alters the mRNA levels of *ZNF703* gene while inducing apoptotic cell death in breast cancer-derived cell lines. The use of *ZNF703* suppression can be considered as a beneficial target in breast cancer research.

Keywords: Apoptosis, and Proliferation, Breast cancer, Invasion, Zinc finger protein.

Introduction

"Cancer" is generally referred to a group of diseases that involve abnormal cell growth and invasion of adjacent or distant cells or tissues. The development of new anti-cancer drugs remains extremely challenging due to tumor heterogeneity and other possible related factors (1). Breast cancer is the most common cancer in women in the USA, with approximately 200,000 new cases and 40,000 deaths annually (2, 3). Recent studies have established various molecular breast cancer subtypes (4, 5), and new drugs that effectively target tumor cell growth have been developed. Determination of estrogen receptor (ER) status is considered to be a useful prognostic factor in the management of malignant breast carcinoma (3). Some investigations identified the roles of novel anti-hormonal factors in the hormone receptor-positive patients (6) and ''triple-negatives," such as HER-2, ER^-, and progesterone-ER^- patients (7). Although treatments somewhat improved survival rates in early-diagnosed HER-2-positive breast cancer patients, these treatments were not effective in triple-negative breast cancer patients.

A growing number of studies over the past twenty years has focused on naturally-occurring compounds that induce programmed cell death

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(apoptosis) in breast cancer cells (8). The understanding of apoptosis has contributed to new anti-cancer treatments (9). Cancer cells evade the apoptotic program by many mechanisms (10). Zinc finger genes (*ZNFs*), an essential gene family in all mammalian species, are thought to influence downstream gene expression by facilitating the interactions between regulatory proteins and DNA sequences (11). *ZNFs* are also believed to have fundamental functions in physiological processes and pathways, such as development and cell proliferation, but the exact underlying mechanisms have not yet been fully characterized.

Recent studies revealed that natural resources represent a substantial segment of the pharmaceutical market (12). Among many herbal plants currently under investigation for their apoptotic properties in humans, *Levisticum (L.) officinale* Koch is a towering perennial aromatic plant from the Apiaceae (Umbelliferae) family found in many European and East Asian countries (13). The Umbelliferae family comprises several species that traditionally have been used in herbal medicine (14). The primary secondary metabolites of *L. officinale* are coumarin, a fragrant organic chemical compound (mostly furano- and pyrano- derivatives), pentylcyclohexadiene, α-terpinyl acetate, polyphenols (phenolic and flavonoid subordinates), essential fatty acids, and specific alkaloids (15, 16). *L. officinale* has spasmolytic and diuretic properties and has been widely used as a medicinal plant for many years (17). This plant is popularly consumed and available natively in Europe, southwestern Asia, and the Sistan and Baluchistan provinces of Iran (18).

Because chemotherapeutic treatments can cause undesirable side effects, natural therapies are often preferred (19). Therefore, determining the effectiveness of herbal products offers promise in cancer treatment. The aim of this study was to evaluate the effects of the hydroalcoholic extract of *L. officinale* on cell proliferation, apoptosis, and gene expression of *ZNF703* (gene ID:80139) also known as *ZNF503L*, *ZEPPO1*, *ZPO1*, and *NLZ1*, in MCF-7 and MDA-MB-468 cells.

Materials and methods

The protocol of the current investigation was approved by the Ethics Committee of Zahedan University of Medical Sciences.

Chemicals

All reagents and biochemicals used in this study were analytical grade and procured as follows: RPMI 1640 medium, Trypan Blue, and trypsin were purchased from INOCLON (G. Innovative Biotech Co. (INOCLON), Iran). The penicillin/streptomycin, fetal bovine serum (FBS), and amphotericin B were purchased from Gibco (Rockville, MD, USA). The Apoptosis Detection Kit was obtained from BioVision (San Francisco, USA). The dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylhiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, USA). The reverse transcription and real-time CR kits were obtained from TaKaRa Bio Inc. (Dalian, China).

Plant material

L. officinale plants were field grown during the spring and summer of 2017 and collected from southwest areas of Sistan and Baluchistan provinces of Iran. Samples were authenticated taxonomically by members of the Department of Biology, Sistan and Baluchistan University, Zahedan, Iran.

Preparation of hydroalcoholic extract of Levisticum officinale (HELO)

After drying the plants in a dark place, roots and aerial sections were separated and both parts powdered. Using a Soxhlet extractor, the plant powder was extracted at 50 ˚C over 6 h with 350 mL of 70% ethanol, followed by filtration with Whatman No. 41 filter paper (20). Then the extract was dried at 45 ˚C using a centrifugal evaporation unit (MAXI DRY-LYO, Denmark). The extract was weighed, dissolved in 1 mL DMSO, and stored at -20 ˚C until further use.

Cell Lines and cell treatment

MCF7 and MDA-MB-468 cells were obtained from the National Cell Bank of Iran (NCBI),

Tehran, Iran. The cells were cultured in RPMI 1640 culture medium supplemented with 100 U/mL streptomycin, 105 mg/mL penicillin, 2.5 mg/L amphotericin B, and 10% FBS under 95% humidified air and 5% CO_2 at 37 °C. Cells were seeded in 6- or 96-well plates 24 h before treatment. After 24 h, the medium was replaced with various concentrations of HELO in equal volumes of FBS-containing medium with less than 1% DMSO as the solvent. All experiments were performed in triplicate.

Cell Growth Inhibition Assay

Following the overnight incubation, 4500 cells/well were seeded in wells of 96-well plates. When the cells reached approximately 80% confluency, MTT assays were performed to determine the IC_{50} of HELO on both cell lines. Extracts of increasing HELO concentrations were added to each well and cells were incubated for 24, 48, or 72 h. At the end of each incubation period, 20 µL of tetrazolium dye at 5 mg/mL was added, and the cells were incubated at 37 °C for 2 h. Later, the culture medium was removed, 180 µL of DMSO was added, and the absorbance at 570 nm was measured using a Stat Fax 2100 microplate reader (Awareness Technology, Inc, USA).

Apoptosis Assay

Cells were seeded at 1 x 10^5 cells/well in 6-well plates and incubated 24 h before treatment with increasing concentrations of HELO. After 48 h of HELO treatment, cells were trypsinized, washed two times with cold phosphate-buffered saline (PBS), and resuspended in PBS. The annexin V/PI Double Staining Kit was used for calculating early and late apoptotic percentages and necrotic cells, according to the manufacturer's instructions. Briefly, the cell pellets were stained in 250 µL of 1x binding buffer and then incubated with 2.5 µL of Annexin V in the dark at room temperature for 15 min. Next, 2.5 µL of propidium iodide was added and the cells were incubated for 10 min. Finally, 100 µg/mL of working solution was added, and the samples were analyzed on a fluorescence-activated cell sorting (FACS) cytometer.

Gene expression assay

Primer design

ZNF703 sequences were downloaded from NCBI and searched online against NCBI sequences with the BLAST family of programs. The designed *ZNF703* forward (F) and reverse (R) primer sequences were 5-GTCCTCCACTCCCGTCAG-3 and 5-CCACCGAGTTGAGTTTGGAG-3, respectively, in addition to GAPDH primers (F: 5-CATGTAGTTGAGGTCAATGAAGG-3, R: 5-GAGCCACATCGCTCAGACAC-3), which was believed to be stably expressed and employed as a reference. The primer efficiencies were verified by constructing standard curves through serial dilutions.

Total RNA Extraction and cDNA Synthesis

After seeding and overnight incubation at 37° C, cells were treated with IC_{50} of HELO and incubated for 0, 2, 4, 8, 12, and 24 h. The cells were trypsinized, rinsed with one mL of cold phosphate-buffered saline (PBS), and aliquotted into 1.5 mL micro-tubes. One mL of RNX (SinaClon, Tehran, Iran) was added to each micro-tube, and total RNA was extracted from cells harvested immediately after each incubation time. The RNA was precipitated and resuspended in 30 µL of DEPC water. UV spectrophotometry at wavelengths of 260 and 280 nm (A 260/A 280) was performed to evaluate the quantity and purity of the extracted RNA. To synthesize cDNA, 4 µg of template RNA from the time 0 [control], 6, 12, or 24 h harvests, and 0.5 µL of random 6-mers (50 µM), 0.5 µL of RNase Inhibitor (40 U/µl), and 2.5 µL RNase-free dH2O were mixed in a microtube. After incubation for 5 min at 65 °C, 0.5 µL of PrimeScript™ RT enzyme Mix I and 2 µL of 5X PrimeScript™ Buffer were added for a final volume of 10 µL. The cDNA synthesis conditions were 37 °C for 10 min followed by 45 °C for 50 min and 95 °C for 5 min. cDNA samples were stored at –20 °C.

Real-time PCR

The Real-Time PCR ABI Sequence Detection System (Applied Biosystems, Foster City, CA,

USA) was employed to determinate *ZNF703* mRNA expression in triplicate based on the manufacturer's protocol. A non-template control was included in all batches. The qRT-PCR amplification reaction mixture contained 1 µL each of both reverse and forward primers (10 pmol), 2 µL of cDNA, 10 µL of SYBR Green EXTaq II PCR Master Mix (2X) and 6 µL of DEPC water. The PCR program began with a primary denaturation at 96 °C for 9 min, followed by 40 cycles of 96 °C for 35 s, 58 °C for 35 s, and 72 °C for 45 s. The $2^{-\Delta\Delta Ct}$ method was applied to determine the mean difference between the expression levels.

Statistical Analysis

The statistical analysis used the Windows version of SPSS package software (release 22, SPSS Inc., USA). The data were analyzed using a non-parametric analysis of variance (ANOVA) between the groups. One-way ANOVA and post-hoc Tukey's tests were used to calculate the importance of differences. P values less than 0.05 were considered significant.

Results

MTT assay

In the MTT test for measurement of the IC_{50}, cell lines were exposed for 24, 48, or 72 h with different *L. officinale* HELO concentrations. The IC_{50}s for the ER^+ (A) and ER^- (B) cell lines were 200 µg/mL and 150 µg/mL, respectively, after 48 h. As shown in Fig.1, the treatment of MDA-MB-468 cells and MCF-7 cells with the extract induced concentration- and time-dependent cell death.

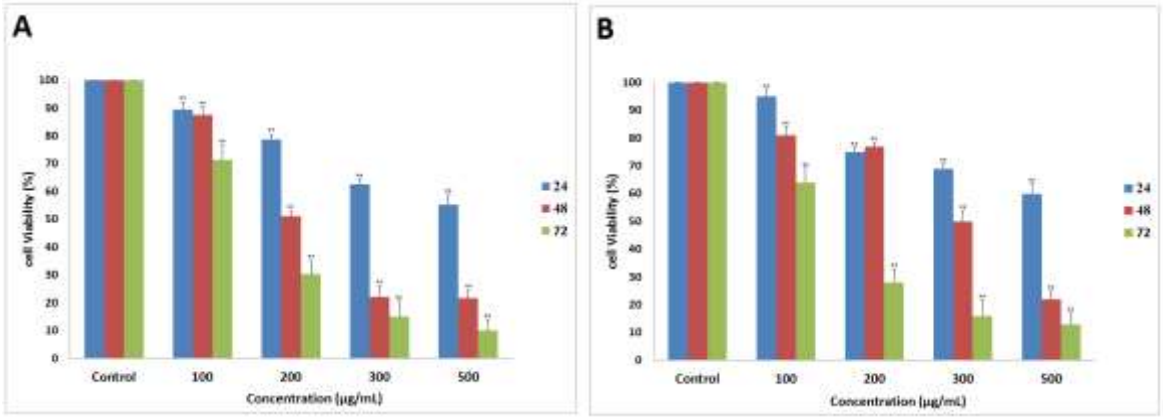

Fig. 1. Cytotoxic effects of *Levisticum officinale* hydroalcoholic extract (HELO) on breast cancer cell lines. Cells were incubated for 24, 48, or 72 h with 0, 100, 200, 300, or 500 µg/mL of *Levisticum officinale* hydroalcoholic extract. Cell viability was measured using MTT assays. Treatment of MDA-MB-468 cells and MCF-7 cells with the extract induced concentration- and time-dependent cell death with IC_{50}s about 200 µg/mL and 150 µg/mL for MCF-7 and MDA-MB-468 cells respectively, after 48 h treatment. **P < 0.05 shows significance relative to untreated controls.

Analysis of apoptosis

To determine whether HELO induced apoptosis, the MCF-7 cells (A)were treated for 48 h with 0, 100, 200, or 300 µg/mL of HELO, while the MDA-MB-468 cells (B) were treated for 48 h with 0, 50, 100, 150, or 300 µg/mL of HELO. The cells were double-stained with annexin V and PI and examined by flow cytometry. Both early and late apoptosis were significantly increased in both cell lines following HELO treatment (Fig.2).

ZNF703 mRNA expression

ZNF703 mRNA was detected in both cell lines, with its expression being 71.43-fold greater in MDA-MB-468 than in MCF-7 cells (Fig.3). Cells were treated for 0, 2, 4, 8, 12, or 24 h with their respective IC_{50} concentrations of 200 µg/mL for MCF-7 and 150 µg/mL for MDA-MB-468, and mRNA was measured by real-time PCR. Significantly less levels of *ZNF703* mRNA was found in both cell lines after 4, 8, 12, and 24 h of HELO treatment than in untreated cells (Fig. 4).

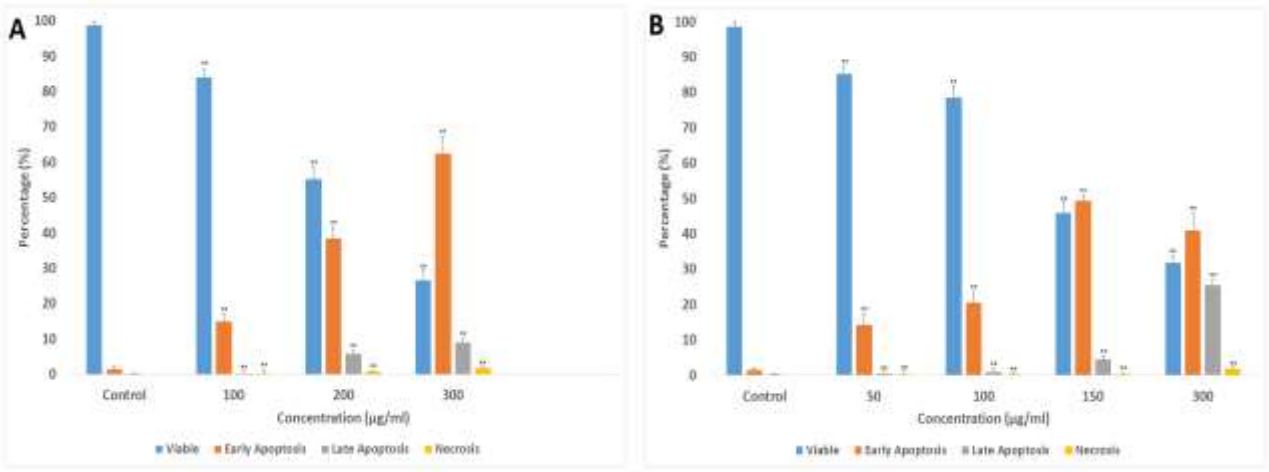

Fig. 2. Analysis of apoptosis in MCF-7 and MDA-MB-468 cells after incubation with *Levisticum officinale* hydroalcoholic extract (HELO). Cells were incubated for 48 h with various concentration of HELO in (A) MCF-7 and (B) MDA-MB-468 cells. Apoptosis was analyzed by flow cytometry. Both early and late apoptosis were significantly increased in both cell lines following the treatment. **P < 0.05 shows significance relative to untreated controls.

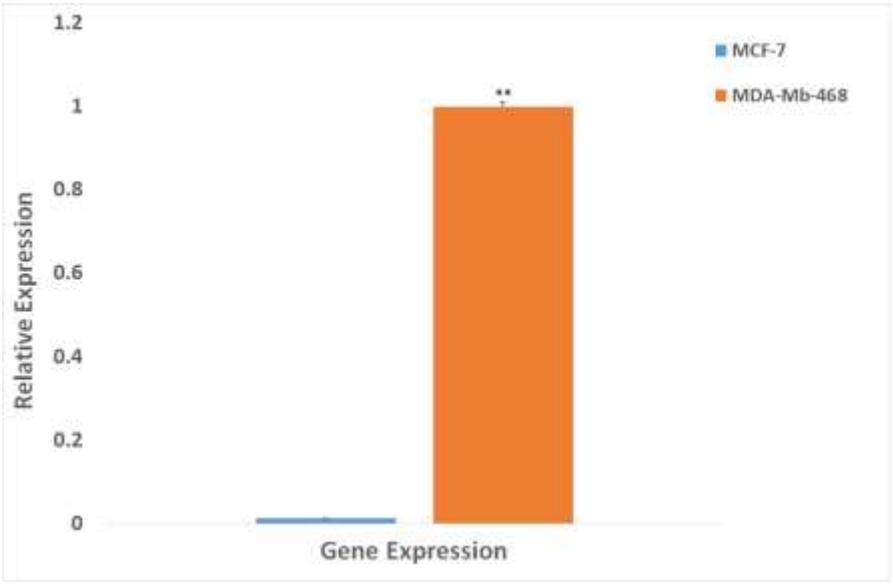

Fig. 3. *ZNF703* expression in MCF-7 and MDA-MB-468 cells. Total RNA was extracted from untreated cultured cells, cDNA was synthesized, and real-time PCR was performed to determine the relative amounts of mRNA in the two cell lines. *ZNF703* mRNA level is measured to be 71.43-fold higher in MDA-MB-468 than in MCF-7 cells (**P < 0.05).

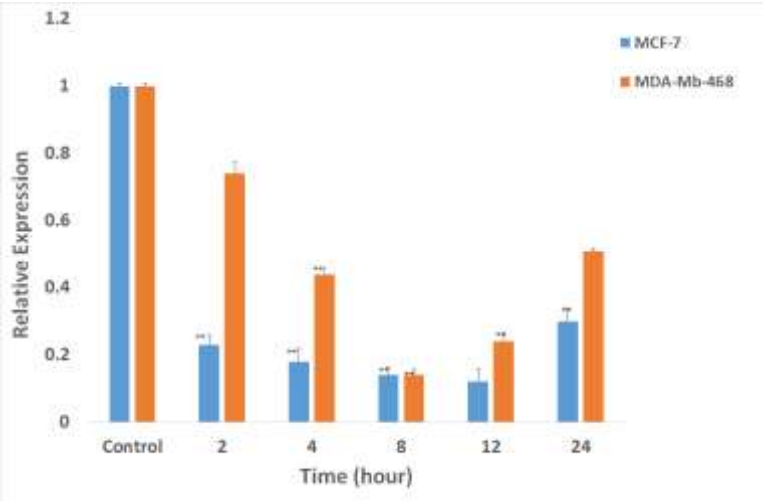

Fig. 4. *ZNF703* expression in breast cancer cell lines. Cultured MCF-7 and MDA-MB-468 cells were incubated with 200 or 150 µg/mL of *Levisticum officinale* hydroalcoholic extract (HELO), respectively, for 0, 2, 4, 8, 12, or 24 h. After each time point, total RNA was extracted, cDNA was synthesized, and real-time PCR was performed to determine the amounts of mRNA in the two cell lines relative to their respective controls. Significantly less *ZNF703* mRNA was found in both cell lines after 4, 8, 12, and 24 h of HELO treatment than in untreated cells (**P < 0.05).

Discussion

ZNF703 is located on 8p11.23 with two exon regions. The gene product is an oncogenic transcription factor that affects several cancer characteristics including growth, enhanced self-renewal, and progression (21). Breast cancers are often associated with chromosome 11q12-14 and/or 8p12 amplifications. *CCND1*, which encodes cyclin D1 at 11q13, induced expression of *ZNF703* mapping at 8p12. These two genes, together with a dominant-negative *TP53* mutant, increased colony formation in breast cancer cells (22). In another study, *ZNF703* mRNA expression was increased in a luminal B breast cancer subtype (23). *ZNF703* has also been linked to cells malignant properties including decreased cell-cell adhesion and increased invasion, proliferation, and metastasis (24). Based on the critical roles of *ZNF703* in cancer progression, especially in breast cancer, we propose that the suppression of *ZNF703* expression leads to reduced breast cancer cell proliferation. Furthermore, *L. officinale* is an anti-proliferative and apoptosis-inducing agent that down-regulates of *ZNF703* expression. In this work, we examined the effect of *ZNF703* inhibition on MFC-7 (ER^+) and MDA-MB-468 (ER^-) proliferation by treating cells with various HELO concentrations.

ZNF703 was expressed in both cell lines; however, its expression in MDA-MB-468 cells was significantly higher than in MCF-7 cells. Two independent studies indicated that MDA-MB-468 cells were more invasive than MCF-7 cells (24, 25); moreover, *ZNF703* is strongly associated with invasion. Also, HELO was able to effectively suppress *ZNF703* expression in treated periods with the inhibitory effect of being greater in the MCF-7 than in the MDA-MB-468 cells, but the most significant inhibition was observed at 8 h in both cell lines. Also, both early and late apoptosis were induced by HELO in MCF-7 and MDA-MB-468. In agreement with our findings, Sertel et al. showed the essential oil from *L. officinale* leaf extract had anti-proliferative activity against a head and neck squamous carcinoma cells (UMSCC1) (26).

Our results demonstrated that HELO not only inhibits *ZNF703* expression and cell proliferation but also induces apoptosis in both ER^+ and ER^-breast cancer cell lines. These results suggest that that HELO may be an effective breast cancer treatment. Further investigations are required to verify and expand on our findings.

Acknowledgment

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