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Original Article

Anti-Tumor Effects of the Methanol Extract of *Ecballium elaterium* (L.) A. Rich Fruits on Human Gastric (MKN-45) and Breast (MCF-7) Cancer Cell Lines

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Abstract

Background and Aim: The *Ecballium elaterium* (L.) A. Rich belongs to the Cucurbitaceae family and is a Mediterranean medicinal herb with a medicinal background. In the present study, following our previous research, we aimed to evaluate the expression profile of certain significant genes involved in the process of apoptosis in human gastric (MKN-45) and breast (MCF-7) cancer cell lines treated with the methanol extract of *E. elaterium* fruits.

Materials and Methods: The real-time PCR method was employed to calculate the expression levels of p53, bax, and bcl-2 genes in both MKN-45 and MCF-7 cell lines. Moreover, the bax/bcl-2 mRNA ratio was calculated in both cell lines.

Results: Real-time PCR revealed that *E. elaterium* fruit extract-treated MKN-45 cells underwent a strong reduction in the mRNA levels of bcl-2 (0.04-fold) (p<0.0001) together with an increase in p53 (18.23-fold) (p<0.0001) and a significant reduction in bax (0.31-fold) (p<0.01). However, the up-regulation of bax (55.51-fold) (p<0.0001) and bcl-2 (17.68-fold) (p< 0.05) genes with a concomitant revealed no significant result for p53 (2.32-fold) gene observed in MCF-7 cells. This extract had strong apoptotic activity on MKN-45 cells. The bax/bcl-2 mRNA ratio in *E. elaterium* fruit extract-treated MKN-45 cells was significant in comparison with the control group (7.24-fold higher and p= 0.0133), but this ratio was not remarkable in *E. elaterium* fruit extract-treated MCF-7 cells compared with the control group (3.95-fold higher and p= 0.0811).

Conclusion: The results of this research indicated that *E. elaterium* fruit extract could be a promising therapeutic option against gastric cancer.

Keywords: *Ecballium elaterium* extract, Breast cancer, Apoptosis, p53, bcl-2, Bax

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Introduction

Cancer described by uncontrolled cell multiplying is considered as one of the major causes of mortality in humankind (1). For instance, breast and gastric cancers have high rates of mortality (2-4). Breast cancer appropriates the most usual type of neoplasm in women all over the world (5-7). On the other hand, gastric cancer takes fifth place throughout the world (4, 8, 9). Despite the development of cancer therapy, drug resistance remains a major limitation. Hence, researchers are investigating more secure ways such as the use of medicinal herbs that might contribute to the treatment of cancers (2, 5).

Statistics corresponding to the World Health Organization (WHO) have indicated that medicinal herbs are being used by many people in many countries (80%) in the healing of several disorders (10). Numerous studies have indicated that several medicinal herbs possess certain compounds with anti-tumor activity such as phenol, flavonoid, and alkaloids. Due to such a feature, researching on these herbs is increasing (11-13). Medicinal herbs-derived agents play a key role in cancer treatment (14). For instance, etoposide, a semi-synthetic compound of epipodophyllotoxin (extracted from the rhizome of *Podophyllum peltatum*), has been commended by FDA as a remedy of certain types of cancers (12, 15).

The death of cancerous cells is the chief target in cancer treatment occurred by apoptosis or programmed cell death. Apoptosis, which is a process highly regulated by some genes, can be initiated through either the death receptor (extrinsic) or the mitochondrial (intrinsic) pathway (16, 17). The Bcl-2 (B cell lymphoma-2) family is the vital apoptosis regulator in the intrinsic pathway. It can be classified into the anti-apoptotic (e.g. Bcl-2) and pro-apoptotic (e.g. Bax) members (18, 19). Cell proliferation and apoptosis arrest occur upon p53 inactivation (20). P53 is a pro-apoptotic tumor suppressor protein (21). Up-regulation of anti-apoptotic or down-regulation of pro-apoptotic proteins have been demonstrated in numerous cancers (20, 22). *Ecballium elaterium* (L.) A. Rich (aka squirting or wild cucumber) in the family Cucurbitaceae is a Mediterranean medicinal herb (1, 23, 24). *E. elaterium* has long been utilized in traditional treatment owing to its diverse therapeutic properties in diseases such as sinusitis, liver disorders, rheumatic diseases, and fever (25, 26). There are several reports of anti-inflammatory, antimicrobial, cytotoxic, anticancer, immunomodulatory, and hepatoprotective effects of this plant (24, 27). For instance, the results of an investigation by Bohlooli *et al*. (28) indicated that the aqueous extract of the fruits of *E. elaterium* had cytotoxic outcomes on human stomach adenocarcinoma cell line (AGS) via apoptosis. Several reports have attributed the antitumor activity of extracts of *E. elaterium* to cucurbitacins, its effective chemicals (1,9, 29).

In our previous study, we evaluated the antioxidant, antibacterial and cytotoxic activities of the methanol extract of *E. elaterium* leaves and fruits on cancer cell lines, including MCF-7 and MKN-45 (30). As far as our investigation has revealed, no study has been conducted on the effect of *E. elaterium* extracts on the expression of p53, bax, and bcl-2 genes on MCF-7 and MKN-45 cell lines. With due attention to these documents, the objective of this experiment was to analyze the impact of the methanol extract of *E. elaterium* fruits on the expression of mentioned genes by real-time PCR method. Here we report that methanol extract of *E. elaterium* fruits can trigger apoptosis in MKN-45 and relatively in MCF-7 cells.

Materials and Methods

Ethical Considerations

This study was approved by the Ethical Committee of Guilan University of Medical Sciences (ID: IR. GUMS. REC. 1396. 247, Date: 6.10.2018).

Plant Materials

This study follows our previous research regarding the assessment of antioxidant, antibacterial, and cytotoxic activities of the methanol extract from fruits and leaves of *Ecballium elaterium* (L.) A. Rich (30). For the extraction of methanol, the leaves and fruits of the plant (300 g each) were dried in the shade and ground to a powder. Subsequently, they were extracted by percolation with methanol at room temperature for 24, 48 and 72 h. The obtained solvent was evaporated by rotary evaporator to achieve the methanol extract of leaves (61.8 g) and fruits (26.5 g). The extracts were

preserved in a refrigerator until further use.

IC_{50} Calculation

As it was mentioned in our previous report (30), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was carried out based on the manufacturer protocol. Then, the IC_{50} (median growth inhibitory concentration) values for the fruit extract of the plant were calculated based on our previous report (30) for two cancerous cell lines, including MCF-7 and MKN-45. Briefly, using an ELISA reader device (Stat Fax 2100, Awareness, USA), the absorbance was measured at 570 nm and the cell survival was evaluated. The concentration-response curve in Microsoft Office Excel (2010) was used to determine the IC50 values (median growth inhibitory concentration). Each data was the mean value of three individual trials and presented as mean ± SD. All the experiments were performed in triplicate.

Cell culture

With regard to the achievement of enough RNA for cDNA synthesis and gene expression examination by real-time PCR, the selected cell lines (MCF-7 and MKN-45) were cultured in 6-well plates. All the cell lines were purchased from the Pasteur Institute, Tehran, Iran. The cancerous cell lines were cultured in RPMI1640 medium (Gibco, Germany), supplemented with 10% fetal bovine serum (FBS, Gibco), and 1% penicillin G-streptomycin (10000 Units/mL, Sigma-Aldrich, Sweden), at 37 °C in humidified air containing 5% CO_2. In short, 1×10^6 (cells of each cancerous cell line/well) was plated in 6-well plates including the calculated IC_{50} for fruits of the plant. Then, the cells were incubated at 37 °C, in 5% CO_2, for 24 h at a humidified atmosphere. It is worth mentioning that next to each of the wells for the extract, a well from the same cell line was cultured as the control (without the addition of the extract).

RNA Extraction and cDNA synthesis

RNA Extraction was performed using YTzol Pure RNA solution (YEKTA TAJHIZ AZMA, Iran) according to the manufacturer's procedure. The quality and quantity of the extracted RNA were evaluated by a NanoDrop spectrophotometer (31, 32), (NanoDrop 2000, Fisher Scientific, USA) and about 1000 ng/µl of the extracted RNAs was used for cDNA synthesize using a cDNA Synthesis kit (Yektatajhiz Azma, Iran) according to the manufacturer's protocol (13).

Real-Time PCR

Expressions of p53, bax, and bcl-2 genes and GAPDH (as housekeeping gene) were examined by SYBR Green Real-time PCR dye using Applied Biosystems Step One™ Real-Time PCR System (USA). Primer3web (version 4.0.0) was used for designing the primers and was ordered to GenFanAvaran Co.; Tehran, Iran. Table 1 shows the details of the designed primers. The concentration was 10 pmol/mL for primers. The reaction mixture included 1 µl of each primer, 4 µl of diluted cDNA, 10 µl of SYBR Green Master Mix (YEKTA TAJHIZ AZMA, Iran), and 4µl nuclease-free water. The PCR condition was a pre-activation stage of 15 min at 95°C (initial denaturation), followed by 40 cycles of 15 secs at 95°C, and 60 secs

Table 1: Sequences of gene-specific primers.

Primer name	Sequence (5'→3')	Product size
GAPDH-F	GACAGTCAGCCGCATCTTCT	104
GAPDH-R	GCGCCCAATACGACCAAATC	
p53-F	GTGGAAGGAAATTTGGGTGTGG	184
p53-R	CCAGTGTGATGATGGTGAGGATG	
bax-F	TCTGACGGCAACTTCA	186
bax-R	GAGGAGTCTCACCCAACCAC	
bcl-2-F	TGCACGTGACGCCCTTCAC	293
bcl-2-R	AGACAGCCAGGAGAAATCAAACAG	

at 60°C. Moreover, the bax/bcl-2 mRNA ratio was measured in MKN-45 and MCF-7 cells treated with or without *E. elaterium* fruit extract as a valid apoptosis index. The reactions were run in duplicate for each sample. Finally, the differences in the expressions of p53, bax, and bcl-2 mRNAs in the samples were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The statistical investigation was carried out by means of GraphPad Prism 8.3.0 software (GraphPad Software, USA). Variations between the two groups were calculated by unpaired t-test. p <0.05 was taken into consideration as statistically significant.

Results and Discussion

IC_{50} values

The calculated IC_{50} values for fruits of the plant-based on our previous study (30) was 2062 µg/ml (MCF-7) and 1960 µg/ml (MKN-45).

Evaluation of the mRNA Expression of p53, bax, and bcl-2 Genes

The expression of apoptosis-related genes in MCF-7 and MKN-45 cells treated with the methanol extract of *E. elaterium* fruits in IC_{50} values was evaluated by real-time PCR after 24 h. Significant up-regulation of p53 (18.23-fold) and a remarkable decrease in bax (0.31-fold) mRNA levels were associated with noticeable down-regulation of bcl-2 (0.04-fold) in MKN-45 cells compared to the control ($p < 0.0001$, p <0.01 and $p < 0.0001$ respectively) (Figure 1).

There was a substantial increase in the bax (55.51-fold) mRNA expression in MCF-7 cells compared to the control. Nevertheless, p53 expression was not notably up-regulated (2.32-fold) by the treatment of the mentioned extract in this cell line, and bcl-2 levels were increased as well (17.68-fold) ($p < 0.05$ and $p < 0.0001$ respectively) (Figure 2).

The results indicated that the bax/bcl-2 mRNA ratio in the *E. elaterium* fruit extract-treated MKN-45 cells was significant in comparison with the untreated control group (7.24-fold higher and p= 0.0133), but this ratio was not remarkable in the *E. elaterium* fruit extract-treated MCF-7 cells compared to its untreated control group (3.95-fold higher and p= 0.0811) (Figure 3).

In spite of advancements in combating cancer, this disease has still remained the major health issue around the world (33). Apoptosis is an important event in many processes, including cancer cell growth inhibition (34). Due to this feature, inducing apoptosis is a major goal of cancer therapy (16). Although there is a narrow list of medicines with direct effects on apoptosis pathways accepted by the FDA for cancer therapy, the effects of various current anticancer medications are pertaining to the pathways of apoptosis (35). Many natural products originated from medicinal herbs have demonstrated their apoptotic effects on several cancer cell lines as it has been shown in the reported studies. These effects have been shown by the down-regulation of anti-apoptotic or up-regulation of pro-apoptotic genes via

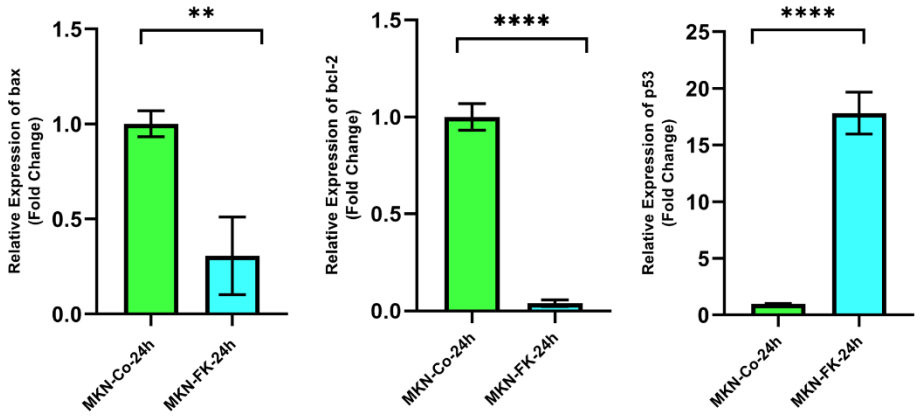

Figure 1. Effects of the methanol extract of *E. elaterium* fruits on the mRNA expression of bax, bcl-2, and p53 genes in MKN-45 cell line. Real-time PCR results showed that the extract caused a strong increase in p53 (right), a slight increase in bax (left) mRNA levels, and a reduction in bcl-2 (middle) expression after 24 h. The data were expressed as mean ± SD. p <0.01**, $p < 0.0001$**** vs. untreated MKN-45 cells as the control.

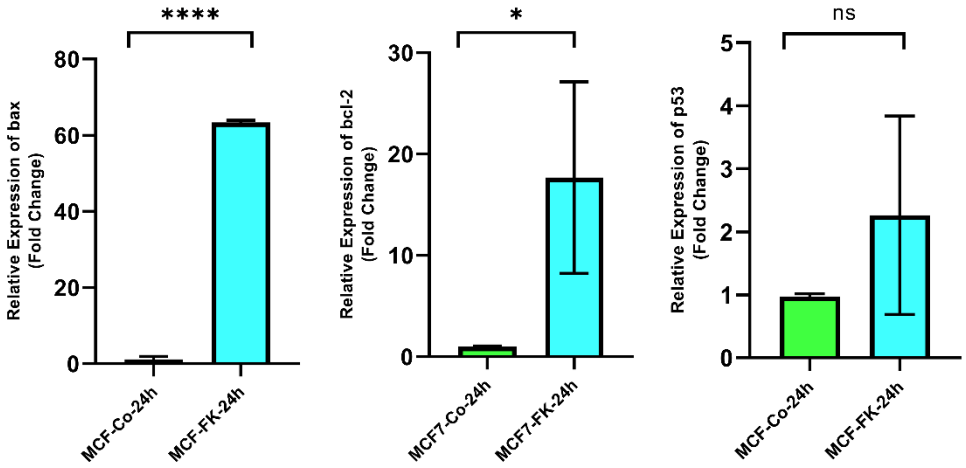

Figure 2. Effects of the methanol extract of *E. elaterium* fruits on the mRNA expression of bax, bcl-2, and p53 genes in MCF-7 cell line. Real-time PCR results showed that the extract strongly increased bax (left) and slightly increased bcl-2 (middle) mRNA levels, while had no remarkable effect was observed on p53 (right) expression after 24 h. The data were expressed as mean ± SD. $p < 0.05$*, $p < 0.0001$****, ns: not significance vs. untreated MCF-7 cells as the control.

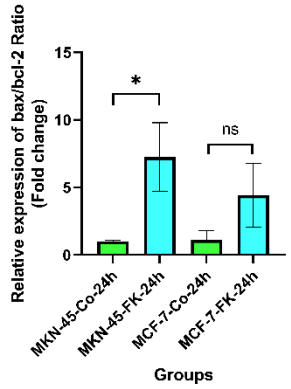

Figure 3. Relative Quantitative Real-time PCR representing the bax/bcl-2 ratio in 4 experimental groups. *E. elaterium* fruit extract treatment resulted in increased bax / bcl-2 ratio in MKN-4 and MCF-7 cells compared to their untreated control groups. The data were presented as mean ± SD. $p < 0.05$*, ns: not significance vs. untreated MCF-7 cells as the control.

certain methods such as real-time PCR (14, 36). Utilization of medicinal herbs is significantly increasing due to less costs as well as less adverse reactions compared to the treatment options currently available(37).

The herb *E. elaterium* has a history of treating diseases such as inflammatory ones.[25] Considerable pieces of evidence of the anticancer and anti-apoptotic properties of *E. elaterium* (L.) A. Rich have been reported in several studies, and since breast and gastric cancers have high rates of frequency and death globally, we studied the consequence of the methanol extract of *E. elaterium* fruits on the expression of major genes involved in apoptosis, i.e. bax, p53 and bcl-2, using real-time PCR method on breast and gastric cancer cell lines. Many studies have been carried out on *E. elaterium* fruit juice than other parts of this plant, including its leaves (25). *E. Elaterium* is a member of the Cucurbitaceae family that produces various types of cucurbitacins (9). Growing evidence has suggested that these substances are responsible for inducing apoptosis in certain cancer cells and have anticancer effects (38). Other properties such as antioxidant, antibacterial and cytotoxic functions of the extracts of this plant have been attributed to a number of secondary metabolites like phenolic, triterpenoids, and flavonoids compounds (30). The proliferation inhibitory effects of *E. elaterium* seed oil on the human colonic adenocarcinoma (HT29) and fibrosarcoma (HT1080) cell lines were observed (39). In a related

survey, the cytotoxic effect of the extract from *E. elaterium* fruits has on gastric adenocarcinoma (AGS) and esophageal squamous (KYSE30) cell lines was investigated (28). The n-hexane extract of the aerial parts of *E. elaterium* and its 100% fraction by Molavi *et al*. (40) displayed strong growth inhibition of MCF-7 cell line (IC50= 264.3 ± 5.2 and 351.2 ± 5.5 µg/mL, respectively). A study identified that cucurbitacins from *E. elaterium* fruits caused cell death in human gastric cancer cell line AGS, although exhibited extremely weak effects on Bax gene by quantitative PCR (4). Moreover, it was observed in another study that cucurbitacin E from cucurbitacin-containing plants increased p53 levels in the human bladder cancer cell line (T24) (41), and in another one Cucurbitacin B enhanced the expression of bax and decreased bcl-2 expression in human osteosarcoma cell line U-2 OS (42). The Bcl-2, as an anti-apoptotic protein, regulates the apoptosis process while P53 and Bax proteins have been considered as pro-apoptotic agents that promote the apoptosis pathway (11). The results of another research revealed increased bcl-2 and decreased bax expression by Cucurbitacin E in 95D lung cancer cells (43).

As mentioned above, the present research attempted to explore, for the first time, the expression of p53, bax, and bcl-2 genes in human breast adenocarcinoma and gastric cancer cell lines MCF-7 and MKN-45 respectively treated with the methanol extract of *E. elaterium* fruits.

MCF-7 cells express estrogen, progesterone, glucocorticoid, and epidermal growth factor (EGF) receptors, and are negative for HER2/neu (44, 45). MKN-45 cells as poorly differentiated adenocarcinoma of the stomach (medullary type) do not express epidermal growth factor receptor (EGFR) while having K-ras wild-type and c-Met oncogene (46, 47). Both MCF-7 and MKN-45 have wild-type p53 gene (48, 49).

The findings obtained from the analysis showed that our extract caused an induction of the apoptosis pathway in MKN-45 cells through up-regulation in the expression of bax (p <0.01) and p53 (p <0.0001) genes and down-regulation in the expression of bcl-2 (p <0.0001) gene as equated to the untreated cells as the control after 24 h. Similarly, there was a marked increment in the expression of bax gene with p < 0.0001 in MCF-7 cells upon the treatment with this extract, but on the contrary an increase was noticed in the expression of bcl-2 gene with p <0.05, and no significant effect on p53 gene was observed in this cell line after 24 h. Furthermore, the methanol extract of *E. elaterium* fruits down-regulated bcl-2 gene only in MKN-45 cells (As it can be seen in Figures 1 & 2, and the data has been expressed as fold change with p <0.05*, p <0.01**, p <0.0001****). Hence, MKN-45 cells displayed a greater sensitivity and responsiveness to the *E. elaterium* fruit methanol extract.

According to the results of this study, treating cancerous cell lines with the *E. elaterium* fruit extract for 24h remarkably increased the bax/bcl-2 ratio in the MKN-45 cells. This ratio, which is considered a reliable index for apoptosis (50, 51), can represent the increase of cell death rate and apoptotic effect of this plant. These findings of the present study are consistent with that found by Russo *et al*. That is, essential oils from three *Salvia* species shifted the Bax/Bcl-2 ratio in favor of apoptosis (52). Lin *et al*. presented a similar result in their study in which Guava (*Psidium guajava* L.) seed polysaccharide fraction 3 (GSF3) increased pro-(Bax)/anti-apoptotic (Bcl-2) mRNA expression ratios in the treated MCF-7 cells (53). Many studies have indicated the apoptotic impact of *E. elaterium* in various cell lines and by particular mechanisms.

In general, this study confirmed that apoptosis has been involved in gastric cell lines treated with the methanol extract of *E. elaterium* fruits. However, further investigations on breast cancer cell line need to be performed in this regard. Interestingly, some of our findings are in accordance with the results of several studies performed on other plants. Bcl-2 down-regulation and bax up-regulation were observed in response to *Euphorbia esula* extract in human gastric carcinoma SGC-7901 cells (54). Previous studies have shown triggering apoptosis in MCF-7 cells via significantly decreased bcl-2 gene expression by the *Calystegia sepium* methanol extract (55) and significant up-regulation of bax and down-regulation of bcl-2 levels by the black turtle bean extract (56). Another study conducted by Patel *et al*. (57) indicated that *Tribulus terrestris* extract could induce up-regulation in the expression of bax and p53 genes and down-regulation of bcl-2 expression.

Conclusion

The findings of the present *in vitro* research confirmed that the methanol extract of *E. elaterium* fruits can trigger the apoptosis process, particularly in MKN-45 cells via the down-regulation of anti-apoptotic bcl-2 and up-regulation of pro-apoptotic p53 and bax. Given the results achieved from this analysis, it might be suggested that the methanol extract of *E. an elaterium fruit* has potential anticancer activity. Nevertheless, more studies need to be carried out in other cancer cell lines to assure the anticancer and anti-apoptotic activities of *E. elaterium* extracts.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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