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Cytotoxic and Apoptosis-Related Effects of Aqueous Trigonella foenum-graecum Seed Extract on SKOV3- Human Ovarian Cancer Cells

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ABSTRACT

Background: Ovarian cancer remains a major therapeutic challenge because of late diagnosis, recurrence, and treatment resistance. Fenugreek (Trigonella foenum-graecum L.) contains several bioactive constituents with potential anticancer properties. This study investigated the cytotoxic effect of aqueous fenugreek seed extract on SKOV-3 human ovarian cancer cells and evaluated its association with the expression of apoptosis-related genes.

Methods: SKOV-3 cells were treated with aqueous fenugreek seed extract at concentrations of 1, 10, 50, 100, and 200 µg/mL for 24 and 48 h. Cell viability was assessed using the MTT assay, and half-maximal inhibitory concentrations (IC50) were determined by nonlinear regression. To investigate apoptosis-related molecular changes, cells were treated with the experimentally determined IC50 concentration, and the relative mRNA expression levels of BAX, BCL2, and CASP3 were evaluated by quantitative real-time PCR (qRT-PCR) using GAPDH as the reference gene. Relative gene expression was calculated using the 2^−ΔΔCt method.

Results: Aqueous fenugreek extract reduced SKOV-3 cell viability in a concentration- and time-dependent manner. The estimated IC50 values were 38.5 µg/mL at 24 h and 19.7 µg/mL at 48 h, indicating greater cytotoxicity with prolonged exposure. Treatment at the IC50 concentration significantly increased BAX expression to 2.83-fold and CASP3 expression to 3.21-fold, while BCL2 expression decreased to 0.43-fold relative to untreated cells. Consequently, the BAX/BCL2 expression ratio increased approximately 6.6-fold, indicating a shift toward a pro-apoptotic molecular profile.

Conclusion: Aqueous T. foenum-graecum seed extract exerted concentration- and time-dependent cytotoxic effects on SKOV-3 ovarian cancer cells and was associated with coordinated modulation of apoptosis-related genes, characterized by increased BAX and CASP3 expression, decreased BCL2 expression, and an elevated BAX/BCL2 ratio. These findings suggest that modulation of apoptosis-related signaling may contribute to the cytotoxic activity of aqueous fenugreek extract. Further functional and protein-level studies are required to confirm apoptosis and clarify the underlying molecular mechanisms.

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INTRODUCTION

Ovarian cancer represents one of the major malignancies affecting the female reproductive tract and contributes substantially to cancer-related deaths in women. Due to non-specific symptoms, diagnosis at advanced stages, invasiveness and metastasis,

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and the development of drug resistance, this disease remains a significant therapeutic challenge. According to GLOBOCAN 2022 estimates, approximately 324,000 new cases of ovarian cancer and over 206,000 associated deaths occurred worldwide in 2022 (1). In Iran, ovarian cancer is also considered a major malignancy in women, and studies based on national data have demonstrated temporal and geographical variations in the incidence and outcomes of female cancers, including ovarian cancer (2, 3). Despite therapeutic advancements, disease recurrence and chemotherapy resistance remain primary causes of treatment failure; consequently, the identification of natural compounds capable of inhibiting cancer cell growth and inducing cell death has garnered significant interest (4).

Fenugreek (Trigonella foenum-graecum L.), a member of the Fabaceae family, produces seeds that have long been utilized for both medicinal purposes and various applications in the food industry. Phytochemical studies have shown that fenugreek contains a diverse range of bioactive compounds, including steroidal saponins, flavonoids, phenolic compounds, alkaloids, and amino acids. Key compounds include diosgenin, trigonelline, and 4-hydroxyisoleucine. These compounds have attracted attention as potential anti-cancer agents due to their antioxidant and anti-inflammatory activities, as well as their ability to modulate cellular pathways. The type of extraction solvent influences the composition and biological activity of the extract; an aqueous extract can extract a significant portion of fenugreek's polar and water-soluble compounds, making it important to directly investigate its effects in cellular models (5, 6).

Evidence from laboratory studies indicates that fenugreek and certain of its active compounds can inhibit proliferation and induce cell death in various cancer models. For instance, fenugreek extract has been shown to induce cell death in Jurkat lymphoma cells (7). Furthermore, saponin compounds found in medicinal plants including those associated with fenugreek can inhibit cancer cell growth by affecting the cell cycle, oxidative stress, and cell death pathways (5). Studies on ovarian cancer cells have also demonstrated that certain steroidal saponins, such as yamogenin, exhibit anti-proliferative effects by inducing oxidative stress, arresting the cell cycle, and activating apoptosis (8). However, it should be noted that the effects of a pure compound cannot be directly extrapolated to the aqueous fenugreek extract; thus, direct investigation of this extract in ovarian cancer cells is essential.

Programmed cell death (apoptosis) plays an essential role in the removal of damaged or abnormal cells, while disruption of this tightly regulated process is recognized as a key hallmark of cancer. In the mitochondrial apoptotic pathway, the balance between proteins of the BCL-2 family plays a pivotal role in determining cell fate. Bax, a pro-apoptotic protein, facilitates the caspase activation pathway by increasing mitochondrial membrane permeability and promoting the release of cytochrome c, whereas Bcl-2 prevents cell death by maintaining mitochondrial integrity (9). Consequently, an increase in Bax expression and/or a decrease in Bcl-2 expression resulting in an elevated Bax/Bcl-2 ratio may be associated with the activation of the mitochondrial apoptotic pathway. Accordingly, alterations in the expression of these two genes can serve as a suitable indicator for investigating the potential role of a plant extract in inducing apoptosis.

Given the existing evidence regarding the bioactive compounds in fenugreek and their potential to modulate cell death pathways, examining the effect of the plant's aqueous extract on ovarian cancer cells could yield valuable insights into its anti-cancer potential. However, evidence concerning the direct impact of aqueous fenugreek extract on ovarian cancer cell viability and its link to changes in key apoptotic genes remains limited. Therefore, the present study was conducted to investigate the effect of the aqueous extract of Trigonella foenum-graecum on ovarian cancer cell viability using the MTT assay and to evaluate Bax, Bcl-2 and CASP3 gene expression via real-time PCR, thereby assessing the extract's anti-proliferative effects and its potential role in inducing apoptosis in these cells.

MATERIALS AND METHODS

Plant material and preparation of aqueous fenugreek seed extract

Fenugreek seeds were sourced from Tehran, Iran. The botanical identity of the plant material was confirmed by a qualified botanist, and a representative voucher specimen was subsequently archived in the designated herbarium.

The seeds were cleaned to remove foreign materials, washed with distilled water, completely dried, and ground into a fine powder using an electric grinder.

The aqueous fenugreek seed extract was prepared using a maceration method based on previously described procedures. Briefly, 50 g of finely powdered fenugreek seeds was mixed with 500 mL of distilled water (1:10, w/v) in a sterile glass container. The mixture was subjected to continuous agitation on an orbital shaker for 12 h at room temperature (approximately 25 °C) to facilitate the extraction of water-soluble constituents. After completion of the extraction process, insoluble plant residues were separated from the mixture by filtration using Whatman No. 1 filter paper. The obtained aqueous extract was then concentrated under vacuum with a rotary evaporator, while the temperature was maintained below 40 °C throughout the process. The concentrated extract was

subsequently subjected to lyophilization to produce a dry crude aqueous extract. Following freeze-drying, the recovered extract was weighed to calculate the extraction yield, placed in an airtight container, and maintained at 4 °C until further use.

The extraction yield was determined based on the weight of the dried extract, which was then kept in a securely sealed container at 4 °C until required for subsequent experiments.

For cell culture experiments, a stock solution of the aqueous fenugreek extract (10 mg/mL) was freshly prepared by dissolving 100 mg of the dried extract in 10 mL of sterile distilled water. Under aseptic conditions, the stock solution was sterilized by filtration through a 0.22-µm membrane filter. Immediately prior to cell treatment, the filtered stock was diluted in complete culture medium to obtain final working concentrations of 1, 10, 50, 100, and 200 µg/mL.

Working solutions of 1, 10, 50, 100, and 200 µg/mL were freshly prepared from the stock solution using complete culture medium.

SKOV-3 cell culture

SKOV-3 human ovarian adenocarcinoma cells were obtained from the Genetic Resources Center in Tehran, Iran. Cells were propagated in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin and incubated at 37 °C under humidified conditions with 5% CO_2. Fresh culture medium was supplied periodically, and cells were subcultured upon reaching approximately 70–80% confluence. Only actively proliferating cells in the logarithmic growth phase were utilized in the experimental assays.

Evaluation of the cytotoxic effect of aqueous fenugreek extract

Experimental design

The cytotoxic effect of aqueous fenugreek extract on SKOV-3 ovarian cancer cells was initially evaluated to determine its half-maximal inhibitory concentration (IC50). Based on a previous study evaluating aqueous T. foenum-graecum extract in SKOV-3 cells, five concentrations were selected: 1, 10, 50, 100 and 200 µg/mL. Untreated cells receiving only complete culture medium were considered the control group (0 µg/mL) (10). Two exposure periods, 24 and 48 h, were investigated independently. Therefore, the experimental groups for each exposure time consisted of: Control (0 µg/mL), 1 µg/mL, 10 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL aqueous fenugreek extract.

MTT cell viability assay

The potential cytotoxic effects of the aqueous fenugreek extract were assessed by the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). SKOV-3 cells were plated in 96-well plates at a concentration of 5×10^3 cells per well. Cells were initially incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2 to ensure adequate attachment. The existing medium was then discarded and substituted with fresh complete culture medium supplemented with aqueous fenugreek extract at final concentrations of 1, 10, 50, 100, or 200 µg/mL. Control cells received complete culture medium without fenugreek extract. Separate plates were prepared for the 24-h and 48-h treatments. At the end of each treatment interval, MTT reagent was introduced into the wells, followed by incubation at 37 °C to promote the formation of purple formazan crystals by viable, metabolically active cells. After incubation, the medium was discarded and the generated formazan was solubilized in dimethyl sulfoxide (DMSO). Optical density (OD) was then measured at approximately 570 nm using a microplate reader. The percentage of viable cells was subsequently determined according to the following formula:

Cell viability (%) = (OD of treated cells / OD of untreated control cells) × 100

The viability of untreated cells was considered 100%. Each concentration was tested in technical replicates, and the experiment was independently repeated at least three times.

Determination of IC50

The percentage of viable SKOV-3 cells was plotted against the logarithm of aqueous fenugreek extract concentration to generate concentration–response curves. The IC50 was defined as the concentration of aqueous fenugreek extract that reduced cell viability to 50% of the untreated control. IC50 values were determined separately for the two exposure periods using nonlinear regression analysis: IC50-24 h and IC50-48 h. The experimentally determined IC50 was subsequently used for the investigation of apoptosis-related gene expression.

Analysis of apoptosis-related gene expression

Treatment of SKOV-3 cells for gene-expression analysis

Following determination of the IC50, a second set of experiments was conducted to investigate whether the cytotoxic activity of aqueous fenugreek extract was associated with modulation of apoptosis-related genes. SKOV-3 cells were seeded into 12-well culture plates at approximately 1×10^5 cells/well, consistent with previously published experiments investigating the effects of fenugreek-derived preparations on SKOV-3 cells. After allowing the cells to attach, the culture medium was replaced with fresh medium. The experimental groups consisted of: Control group: untreated SKOV-3 cells. Treatment group: SKOV-3 cells exposed to aqueous fenugreek extract at the

experimentally determined IC50 concentration. Cells were exposed to the selected treatment before being harvested for RNA extraction.

Total RNA extraction

At the end of the treatment period, the medium was aspirated and the cells were washed with ice-cold phosphate-buffered saline (PBS). Cellular RNA was subsequently extracted with the TRIzol kit (Yekta Tajhiz, Tehran, Iran) following the procedure recommended by the manufacturer. RNA yield and purity were assessed by spectrophotometric determination of absorbance at 260 and 280 nm. Samples that satisfied the required quality and concentration standards were selected for subsequent complementary DNA (cDNA) synthesis.

cDNA synthesis

Equal amounts of total RNA from the different experimental groups were used as templates for cDNA synthesis. Reverse transcription was carried out with a cDNA synthesis kit (Yekta Tajhiz, Tehran, Iran) according to the manufacturer's recommended procedure. The generated cDNA was subsequently stored at −20 °C until qRT-PCR analysis.

Quantitative real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was employed to quantify the relative mRNA expression of genes involved in apoptosis. The genes selected for expression analysis included:

BAX (BCL2-associated X protein) as a pro-apoptotic marker; BCL2 (B-cell lymphoma 2) as an anti-apoptotic marker; and CASP3 (caspase-3) as a key executioner of apoptosis. Gene-specific primers for BAX, BCL2, CASP3, and the selected housekeeping gene were used for amplification. Each qRT-PCR reaction contained cDNA template, gene-specific forward and reverse primers (Table 1), SYBR Green qPCR Master Mix, and nuclease-free water in a final volume according to the manufacturer's recommendations. The qRT-PCR protocol began with a 10-min denaturation at 95 °C, after which amplification proceeded for 40 cycles, each comprising 15 s at 95 °C and 60 s at 60 °C for denaturation and combined annealing/extension, respectively. Upon completion of the amplification cycles, a melting-curve assessment was performed to verify the specificity of the generated amplicons. Gene expression data were normalized against GAPDH as the endogenous reference, and each sample was analyzed in triplicate.

Relative gene-expression analysis

Relative changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. For each sample:

$\Delta Ct = Ct$ (target gene) – Ct (reference gene)

and:

$\Delta\Delta Ct = \Delta Ct$(treated cells) − ΔCt(control cells).

Relative expression was calculated as:

Relative expression = $2^{-\Delta\Delta Ct}$.

Untreated SKOV-3 cells served as the reference group, with their relative gene expression level set to 1. The expression profiles of BAX, BCL2, and CASP3 were then evaluated in treated cells relative to the control group. Furthermore, the BAX/BCL2 expression ratio was determined to provide an additional measure of the relative contribution of pro-apoptotic and anti-apoptotic signaling. An increase in BAX and CASP3 expression accompanied by a reduction in BCL2 expression and an increased BAX/BCL2 ratio was evaluated in the context of activation of apoptotic signaling.

STATISTICAL ANALYSIS

All experiments were independently repeated at least three times, and quantitative results are reported as mean ± standard deviation (SD). For the MTT data, the effects of aqueous fenugreek extract concentration and exposure duration on SKOV-3 cell viability were evaluated by two-way analysis of variance (two-way ANOVA). Dunnett's multiple-comparisons test was subsequently used to evaluate differences between each extract concentration and its respective untreated control. Dose–response relationships were analyzed by nonlinear regression using a four-parameter logistic model. The IC50 values for the 24- and 48-h treatment intervals were estimated independently, together with their respective 95% confidence intervals. For the qRT-PCR data, differences in ΔCt values between untreated SKOV-3 cells and fenugreek-treated cells were evaluated using a two-tailed Student's t-test after

Table 1. Sequences of primers used for each gene in the qRT-PCR reaction.

Gene	Primer seq	TM (°C)	Size (bp)
BAX	F: CCCGAGAGGTCTTTTTCCGAG R: CCAGCCCATGATGGTTCTGAT	62.1 61.9	155
BCL2	F: GGTGGGGTCATGTGTGTGG R: CGGTTCAGGTACTCAGTCATCC	62.6 61.8	89
CASP3	F: GAAATTGTGGAATTGATGCGTGA R: CTACAACGATCCCCTCTGAAAAA	60.0 60.0	164
GAPDH	F: CTGGGCTACACTGAGCACC R: AAGTGGTCGTTGAGGGCAATG	62.0 62.9	101

confirming data normality. The same statistical test was applied to compare the BAX/BCL2 expression ratios between the control and treatment groups. Differences were regarded as statistically significant when $P < 0.05$. GraphPad Prism, version 10.6.1.892 (GraphPad Software, Boston, MA, USA), was used to perform all statistical analyses.

RESULTS

Effect of aqueous fenugreek extract on SKOV-3 cell viability

The cytotoxic potential of aqueous Trigonella foenum-graecum extract was assessed in SKOV-3 ovarian cancer cells using the MTT assay. Cell viability was evaluated following treatment with different concentrations of the extract for either 24 or 48 h. As shown in Figure 1, treatment with aqueous fenugreek extract resulted in a concentration- and time-dependent reduction in SKOV-3 cell viability. The viability of untreated control cells was considered 100%. After 24 h of treatment, exposure to the lowest concentration of fenugreek extract (1 µg/mL) resulted in only a slight reduction in cell viability to 96.4 ± 3.2%. Increasing the concentration to 10 and 50 µg/mL progressively decreased cell viability to 81.7 ± 4.1% and 42.6 ± 3.8%, respectively. At higher concentrations of 100 and 200 µg/mL, cell viability was further reduced to 27.3 ± 2.9% and 14.8 ± 2.1%, respectively. A greater cytotoxic effect was observed following 48 h of exposure. Cell viability was 92.1 ± 3.5% at 1 µg/mL and decreased to 65.4 ± 3.9% at 10 µg/mL. At 50 µg/mL, only 31.2 ± 3.1% of cells remained viable, while treatment with 100 and 200 µg/mL reduced cell viability to 17.6 ± 2.4% and 8.9 ± 1.7%, respectively.

Determination of IC50 values

To determine the IC50 of aqueous fenugreek extract, concentration–response curves were generated from the MTT viability data and analyzed using nonlinear regression. As illustrated in Figure 2, increasing concentrations of aqueous fenugreek extract progressively reduced SKOV-3 cell viability at both exposure times. The concentration–response curve was shifted toward lower concentrations after 48 h compared with 24 h, indicating an increased cytotoxic effect with prolonged exposure. Nonlinear regression analysis yielded an estimated IC50 of approximately 38.5 µg/mL after 24 h of treatment. In contrast, the estimated IC50 after 48 h was approximately 19.7 µg/mL. Thus, prolonging the exposure time from 24 to 48 h reduced the IC50 by approximately 49%, from 38.5 to 19.7 µg/mL.

Effect of aqueous fenugreek extract on apoptosis-related gene expression

To determine whether the cytotoxic activity of the aqueous fenugreek extract involved alterations in apoptosis-related pathways, the relative mRNA expression of BAX, BCL2, and CASP3 was quantified using qRT-PCR. For this analysis, SKOV-3 cells were exposed to the experimentally established IC50 concentration of the aqueous fenugreek extract, whereas cells receiving no extract treatment served as the control group. Target gene expression was

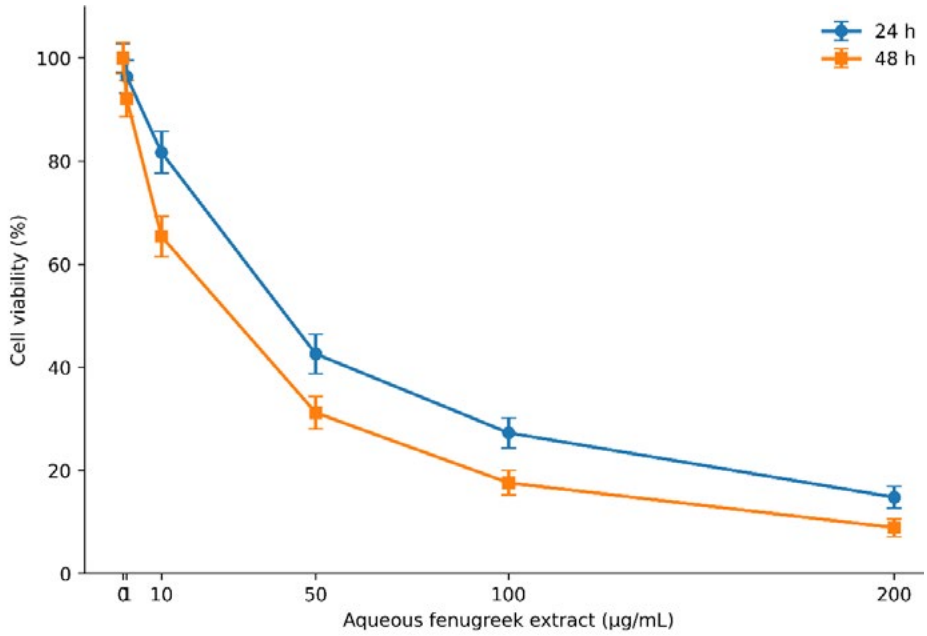


Fig 1. Influence of aqueous Trigonella foenum-graecum extract on the viability of SKOV-3 cells. Cells were exposed to aqueous fenugreek extract at concentrations of 1, 10, 50, 100, and 200 µg/mL for either 24 or 48 h. Cell viability was assessed by the MTT assay and normalized to that of the untreated control group, which was defined as 100% viability. Results are reported as mean ± SD.

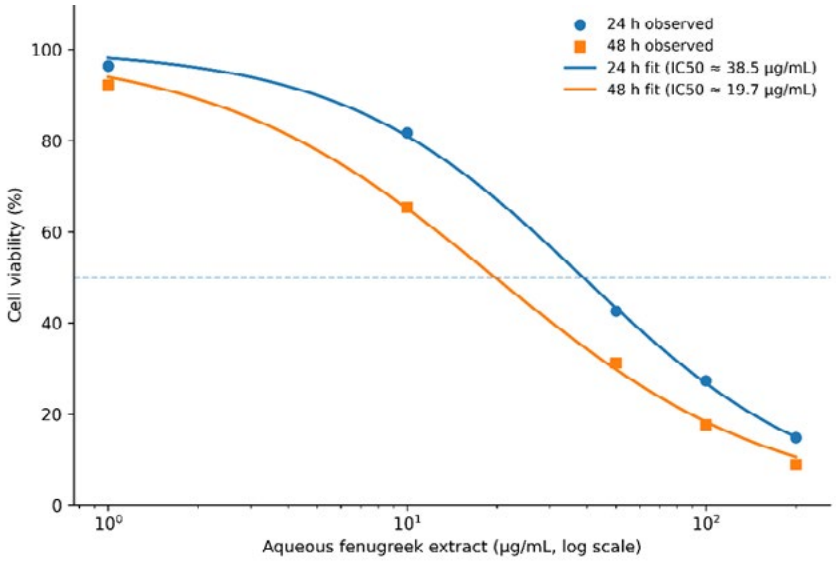


Fig 2. Concentration–response curves and estimated IC50 values of aqueous Trigonella foenum-graecum extract in SKOV-3 cells. SKOV-3 cells were exposed to increasing concentrations of aqueous fenugreek extract for 24 and 48 h. Cell viability determined by MTT assay was fitted using nonlinear regression. The horizontal dashed line represents 50% cell viability. The estimated IC50 values were approximately 38.5 µg/mL for 24 h and 19.7 µg/mL for 48 h.

normalized to the endogenous reference gene, and fold changes in mRNA levels were determined according to the $2^{-\Delta\Delta Ct}$ approach. For comparison, the expression of each target gene in the untreated control group was defined as 1.0.

BAX expression

Exposure of SKOV-3 cells to the aqueous fenugreek extract significantly enhanced the expression of the pro-apoptotic gene BAX. The relative expression level of BAX reached 2.83 ± 0.27-fold in extract-treated cells, compared with 1.00 ± 0.11-fold in the untreated control group. Thus, fenugreek treatment induced an approximately 2.8-fold elevation in BAX expression, representing a statistically significant difference relative to untreated cells ($P < 0.01$).

BCL2 expression

In contrast to BAX, treatment with aqueous fenugreek extract significantly reduced expression of the anti-apoptotic BCL2 gene. Relative BCL2 expression decreased from 1.00 ± 0.09-fold in untreated SKOV-3 cells to 0.43 ± 0.07-fold following treatment with the IC50 concentration of aqueous fenugreek extract. Thus, BCL2 mRNA expression was reduced by approximately 57% compared with untreated cells ($P < 0.01$).

CASP3 expression

The expression of CASP3, a major executioner caspase associated with apoptotic signaling, was also increased following treatment with aqueous fenugreek extract. Relative CASP3 expression increased from 1.00 ± 0.13-fold in control cells to 3.21 ± 0.34-fold in fenugreek-treated cells. This approximately 3.2-fold increase was statistically significant compared with the untreated control ($P < 0.001$).

The relative expression levels of the apoptosis-related genes BAX, BCL2, and CASP3 in SKOV-3 cells following treatment with aqueous fenugreek extract at the IC50 concentration are presented in Figure 3.

Effect of fenugreek extract on the BAX/BCL2 ratio

Since the balance between pro-apoptotic and anti-apoptotic members of the BCL2 family is an important determinant of cellular susceptibility to apoptosis, the BAX/BCL2 expression ratio was also evaluated. Based on relative gene-expression values, the BAX/BCL2 ratio in untreated control cells was approximately: 1.00, whereas treatment with aqueous fenugreek extract increased the ratio to approximately: 6.58. Thus, treatment resulted in an approximately 6.6-fold increase in the BAX/BCL2 ratio compared with untreated SKOV-3 cells. The marked increase in the BAX/BCL2 ratio was primarily associated with simultaneous upregulation of BAX and downregulation of BCL2 expression (figure 4).

DISCUSSION

In this study, the effects of aqueous Trigonella foenum-graecum seed extract on cell viability and apoptosis-associated gene expression were examined in SKOV-3 human ovarian cancer cells. The results revealed a progressive decline in cell viability as both the extract concentration and duration of exposure

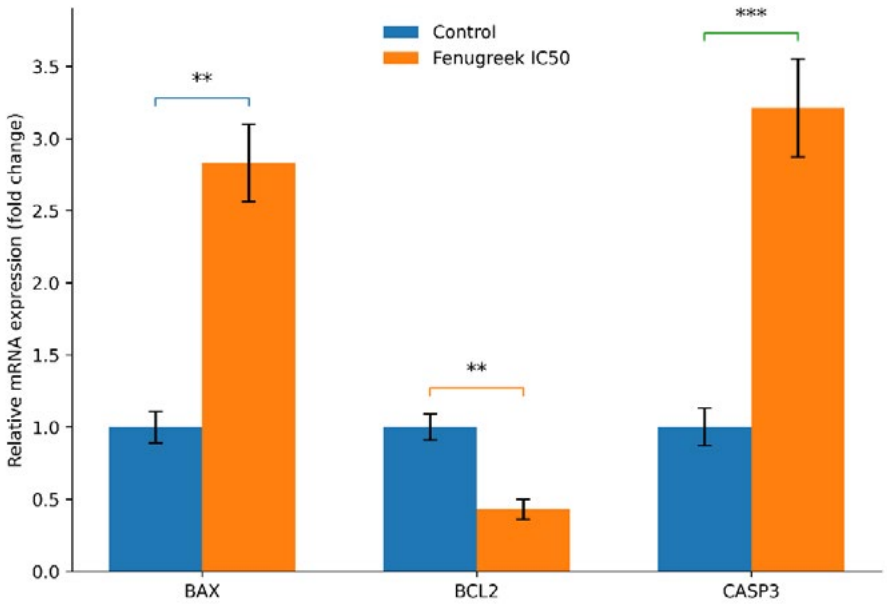


Fig 3. Relative mRNA expression levels of BAX, BCL2, and CASP3 were determined by qRT-PCR and normalized to GAPDH as the reference gene using the 2^-ΔΔCt method.

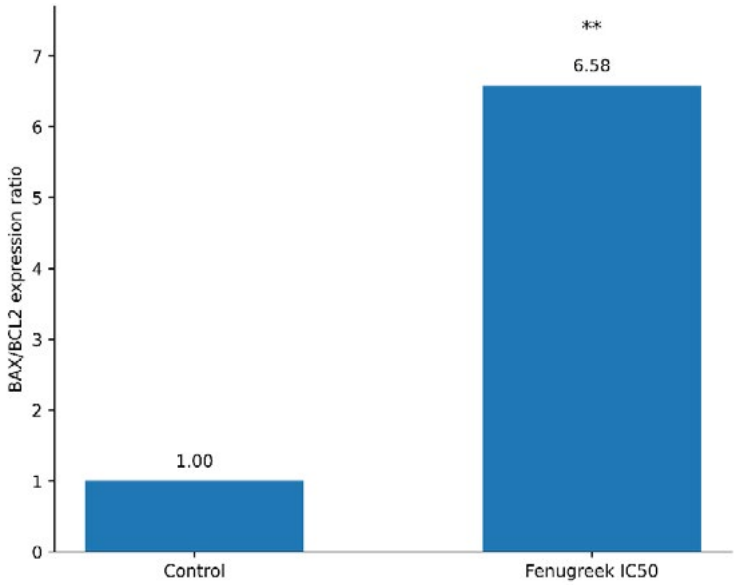


Fig 4. Effect of aqueous Trigonella foenum-graecum extract on the BAX/BCL2 expression ratio in SKOV-3 cells. The ratio of BAX to BCL2 expression was derived from relative mRNA levels quantified by qRT-PCR, with GAPDH serving as the reference gene and relative expression calculated using the 2−ΔΔCt method. Exposure of SKOV-3 cells to the IC50 concentration of aqueous fenugreek extract markedly elevated the BAX/BCL2 expression ratio from 1.00 in the untreated control group to 6.58 in the treated group. This significant increase reflects a shift in apoptosis-related gene expression toward a predominantly pro-apoptotic pattern (**P < 0.01 vs. untreated control).

increased, demonstrating concentration- and time-dependent cytotoxic activity. Moreover, treatment with the experimentally determined IC50 concentration altered the expression pattern of key apoptosis-related genes, as evidenced by increased BAX and CASP3 expression and reduced BCL2 expression. Consistent with these molecular changes, the BAX/BCL2 expression ratio was markedly elevated following treatment. Collectively, these observations indicate that alterations in apoptosis-associated signaling may contribute, at least partially, to the growth-inhibitory effects of aqueous fenugreek extract in SKOV-3 cells.

The MTT assay demonstrated a concentration-dependent decline in cell viability following exposure to the aqueous fenugreek extract. After 24 h of treatment, cell viability decreased from 96.4% at 1 µg/mL to 14.8% at 200 µg/mL, while after 48 h it decreased from 92.1% to 8.9% over the same concentration range. Consistent with these observations, nonlinear regression analysis yielded IC50 values of approximately 38.5 µg/mL

at 24 h and 19.7 µg/mL at 48 h. Thus, extending the exposure time from 24 to 48 h reduced the estimated IC50 by approximately 49%. This finding indicates that prolonged exposure increased the cytotoxic effect of the extract, since a lower concentration was required to produce a 50% reduction in cell viability after 48 h.

The concentration- and exposure time–dependent effects identified in this study are generally in agreement with earlier findings demonstrating the anticancer potential of fenugreek and its biologically active compounds. Of particular relevance, Stefanowicz-Hajduk et al. investigated standardized fenugreek seed extracts, fractions, and secondary metabolites in SKOV-3 ovarian cancer cells and demonstrated cytotoxic activity accompanied by apoptosis-associated cellular responses (11). The agreement between those findings and the present results is important because both studies involve the SKOV-3 cell line, although differences in extract composition and experimental conditions prevent direct numerical comparison of potency. Shokrzadeh et al. also investigated an aqueous preparation of T. foenum-graecum in SKOV-3 cells and demonstrated that these cells are responsive to aqueous fenugreek extract (10). Therefore, the present observation that aqueous fenugreek extract suppresses SKOV-3 viability is supported by previous direct evidence in the same ovarian cancer model.

Additional evidence supporting the susceptibility of SKOV-3 cells to fenugreek-associated compounds was provided by Stefanowicz-Hajduk et al. (2022), who investigated yamogenin, a steroidal sapogenin occurring in fenugreek and other plant species. Yamogenin reduced SKOV-3 viability and induced cellular changes associated with oxidative stress, cell-cycle disturbance, and apoptosis. Although an isolated phytochemical cannot be considered equivalent to a crude aqueous fenugreek extract, these observations provide a biologically plausible link between constituents naturally associated with fenugreek and the cytotoxic response of ovarian cancer cells (8). Fenugreek seeds contain several classes of potentially bioactive compounds, including steroidal saponins and sapogenins, flavonoids, alkaloids such as trigonelline, and other phytochemicals. Therefore, the cytotoxic effect of the aqueous extract observed in the present study may reflect the combined actions of multiple constituents rather than the activity of a single compound.

An important finding of the present study was that the reduction in cell viability was accompanied by marked alterations in apoptosis-related gene expression. Treatment of SKOV-3 cells with the IC50 concentration of aqueous fenugreek extract increased BAX expression approximately 2.83-fold, whereas BCL2 expression decreased to approximately 0.43-fold relative to untreated cells. BAX and BCL2 are functionally opposing regulators of mitochondrial apoptosis. BAX promotes mitochondrial outer membrane permeabilization under pro-apoptotic conditions, whereas BCL2 promotes cell survival by suppressing mitochondrial apoptotic signaling. Therefore, the simultaneous increase in BAX and decrease in BCL2 observed in the present study suggests a shift in the molecular balance toward a pro-apoptotic state.

The direction of these molecular changes is consistent with previous studies investigating fenugreek in other cancer models. Khalil et al. (2015) demonstrated that fenugreek treatment induced apoptosis in HepG2 hepatocellular carcinoma cells and was associated with alterations in apoptosis-related factors, including increased Bax and caspase-3. Similarly, Alrumaihi et al. (2021) reported that methanolic fenugreek seed extract induced apoptosis-associated alterations in breast cancer cells, including mitochondrial depolarization, increased reactive oxygen species generation, and modulation of the Bax/Bcl-2 balance. Although these studies were conducted in different cancer cell lines, their findings support the hypothesis that regulation of mitochondrial apoptosis-related factors may represent an important component of the anticancer activity of fenugreek (12, 13).

The concurrent upregulation of BAX and downregulation of BCL2 markedly shifted the BAX/BCL2 expression ratio, increasing it from approximately 1.0 in untreated SKOV-3 cells to 6.58 after fenugreek treatment. This observation may be particularly relevant because the balance between pro- and anti-apoptotic BCL2-family members can provide more mechanistic information than changes in either gene alone. An increase in the BAX/BCL2 ratio indicates a shift toward pro-apoptotic signaling and may increase cellular susceptibility to mitochondrial apoptotic stimuli. Similar alterations in the Bax/Bcl-2 balance have been reported following treatment of cancer cells with fenugreek preparations (13). Therefore, the marked increase in the BAX/BCL2 ratio observed in the present study provides a plausible molecular link between fenugreek treatment and the reduction in SKOV-3 cell viability.

In parallel with the changes in BAX and BCL2, CASP3 expression increased approximately 3.21-fold following treatment with aqueous fenugreek extract. Caspase-3 is a major executioner component of apoptotic signaling and participates in the cleavage of multiple cellular substrates during the execution phase of apoptosis. Previous investigations have similarly suggested involvement of caspase-associated pathways in the anticancer effects of fenugreek. Khoja et al. (2011) reported that fenugreek induced apoptosis in MCF-7 human breast cancer cells and altered the expression of several apoptosis-associated

genes, including Bax and members of the caspase pathway (14). Khalil et al. (2015) also reported increased caspase-3 in fenugreek-treated HepG2 cells (12). Moreover, the direct investigation of fenugreek-derived preparations in SKOV-3 cells by Stefanowicz-Hajduk et al. (2021) included analysis of caspase-3/7 activity, further supporting the potential involvement of executioner caspases in the response of ovarian cancer cells to fenugreek-derived compounds (11).

Taken together, the coordinated increase in BAX expression, reduction in BCL2 expression, elevation of the BAX/BCL2 ratio, and increase in CASP3 expression are compatible with the involvement of apoptosis-related signaling, potentially involving the mitochondrial pathway. A plausible interpretation is that treatment with the aqueous fenugreek extract alters the balance between pro- and anti-apoptotic BCL2-family members, thereby creating cellular conditions favorable for mitochondrial apoptotic signaling and downstream engagement of the caspase machinery. This interpretation is supported by previous studies in which fenugreek preparations or fenugreek-associated compounds produced mitochondrial alterations, oxidative stress, modulation of Bax/Bcl-2 signaling, and caspase-associated responses in cancer cells (8, 12, 13).

However, the present findings should not be interpreted as definitive evidence of functional activation of the mitochondrial apoptotic pathway. In particular, increased CASP3 mRNA expression does not necessarily indicate activation of caspase-3 protein, since qRT-PCR measures transcriptional abundance rather than proteolytic cleavage or enzymatic activity. Similarly, altered BAX and BCL2 mRNA expression does not directly demonstrate mitochondrial membrane permeabilization. Therefore, the current results are more appropriately interpreted as demonstrating a shift in apoptosis-related gene expression that is consistent with, but does not independently prove, activation of apoptosis. Functional assays such as Annexin V/propidium iodide flow cytometry, caspase-3/7 activity measurement, detection of cleaved caspase-3 and cleaved PARP, mitochondrial membrane potential analysis, or assessment of cytochrome-c release would provide stronger evidence for the proposed mechanism.

Nevertheless, the agreement between the MTT and qRT-PCR findings strengthens the biological interpretation of the present results. MTT analysis demonstrated a progressive decrease in metabolic viability with increasing extract concentration and exposure duration, while qRT-PCR demonstrated a coordinated increase in BAX and CASP3 together with suppression of BCL2. A reduction in MTT signal alone cannot distinguish between apoptosis, inhibition of proliferation, cell-cycle arrest, metabolic suppression, or other forms of cell death. However, when the viability results are considered together with the observed changes in apoptosis-associated genes, an apoptotic contribution to the cytotoxic effect becomes biologically plausible. This interpretation is also consistent with previous fenugreek studies in which reduced cancer-cell viability was accompanied by molecular or functional evidence of apoptosis (8, 11-14).

The greater cytotoxicity observed after 48 h compared with 24 h is also noteworthy. The reduction in the estimated IC50 from approximately 38.5 µg/mL at 24 h to 19.7 µg/mL at 48 h suggests that prolonged exposure increased the apparent cytotoxic potency of the extract. This response may reflect progressive accumulation of cellular stress, sustained suppression of proliferation, or gradual engagement of cell-death pathways. However, numerical differences between IC50 values obtained in the present study and those reported in previous investigations should not necessarily be interpreted as conflicting findings. Extraction solvent, extraction procedure, geographical origin and chemical composition of the plant material, concentration of individual phytochemicals, cancer cell type, initial cell density, duration of treatment, and analytical approach used for dose–response modeling can all substantially influence calculated IC50 values. In particular, aqueous, methanolic, lipid, standardized, and purified preparations of fenugreek are chemically different and should not be expected to demonstrate identical cytotoxic potency.

Overall, the present findings indicate that aqueous T. foenum-graecum seed extract exerts a concentration- and time-dependent inhibitory effect on SKOV-3 ovarian cancer cells and that this response is accompanied by coordinated modulation of apoptosis-related genes. The simultaneous increase in BAX and CASP3, decrease in BCL2, and pronounced elevation of the BAX/BCL2 ratio indicate a shift toward a pro-apoptotic molecular profile. Further studies incorporating functional apoptosis assays, protein-level analysis, additional ovarian cancer cell lines, non-malignant control cells, and phytochemical characterization of the aqueous extract are required to establish the precise molecular mechanisms and selectivity of the observed effects.

CONCLUSION

The results demonstrate that aqueous Trigonella foenum-graecum seed extract exerts cytotoxic effects on SKOV-3 human ovarian cancer cells, with the reduction in cell viability becoming more pronounced as the extract concentration and treatment duration increase. Fenugreek treatment was further accompanied by upregulation of BAX and CASP3, downregulation of BCL2, and a pronounced elevation in the BAX/BCL2 expression ratio, collectively reflecting a shift toward a pro-apoptotic gene expression pattern. These molecular alterations suggest that regulation of apoptosis-associated pathways may be involved in the growth-inhibitory activity of the extract against ovarian

cancer cells. Nevertheless, confirmation of this proposed mechanism requires further investigation incorporating functional assays of apoptosis, assessment of apoptosis-related proteins, additional ovarian cancer cell models, and non-malignant cells. Such studies would also help determine the selectivity and potential anticancer significance of aqueous fenugreek extract.

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Ethics Approval and Consent to Participate

No ethics approval or consent statement is provided in the article. Since the study involved laboratory experiments using a human ovarian cancer cell line (SKOV-3), please confirm whether ethical approval was required and obtained, or whether an exemption applies.

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