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Cell Death Induction and Bax gene expression of Cisplatin and Centaurea behen extract in human breast cancer cell line (MCF-7)

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Background: The uncontrolled growth of the cells in the breast tissue causes breast cancer. About 1.38 new cases of breast cancer are diagnosed worldwide every year. Breast cancer is the most common cancer among women in the United States, with over 266,000 new cases expected for the year 2018. The evolution of breast cancer is a multi-stage process that involves genetic, epigenetic, and environmental factors that may interfere with the main settings of oncogenes and tumor suppressor genes, leading to the activation of signaling pathways associated with cancer that one of the most important of these pathways is the apoptosis process. The Bax gene also acts as an apoptotic stimulant.
Method: Due to the importance of this gene in the apoptotic process, in this research, the expression level of the Bax gene under treatment of cisplatin and Centaurea behen agents for 24 hours and 48 hours was investigated using the Real-time-PCR method.
Results: The results obtained illustrate that the expression level of this gene under treatment with the cisplatin and Centaurea behen has increased compared to the non-treatment state, so this expression increase showed a significant difference between the samples group and control group ($P<0.05$).
Conclusion: We have shown that the Centaurea behen extract and cisplatin could induce apoptosis in the MCF-7 cell line, over-expressing of the Bax gene.
Keywords: apoptosis, breast cancer, Bax, cisplatin, Centaurea behen, quantitative-PCR method

Introduction

The comprehensive management of breast cancer (BC) requires a multidisciplinary approach involving surgical oncology, radiation oncology, and medical oncology. The National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines for Breast Cancer offer clinical management recommendations for patients diagnosed with carcinoma in situ, invasive breast cancer, Paget's disease, Phyllodes tumor, inflammatory breast cancer, and breast cancer during pregnancy, providing a unified framework for optimal patient care.(1,2)

Numerous studies have documented an alarming trend of increasing breast cancer incidence across the globe. Despite the availability of various treatment modalities aimed at preventing and treating the disease, this concerning trend is projected to persist over the next two decades, necessitating persistent research efforts to develop innovative therapeutic approaches.(3) These findings emphasize the pressing need for effective preventive measures, diagnostic tools, and treatment options to combat this formidable disease.(4)Breast cancer remains an epidemic of staggering proportions, affecting millions of individuals worldwide. In the United States alone, one in eight women will be diagnosed with breast cancer at some point in their lives. In 2024, approximately 310,720 women and 2,800 men are projected to receive a diagnosis of invasive breast cancer. (5)

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It is highly likely that you or someone you know has been directly impacted by this disease, underscoring the urgent need for continued advancements in prevention, detection, and treatment.(6) Breast cancer is a complex disease with a multitude of histopathological and molecular sub-forms that exhibit diverse clinical outcomes and risk factor associations. This intricacy warrants a thorough sub-classification of clinical breast tumors, with one prominent classification being based on the presence or absence of estrogen (ER) and progesterone (PR) receptors. (7,8) The identification and characterization of these receptor statuses provide valuable insights into tumor biology, prognosis, and potential therapeutic targets, making them a crucial consideration in the management of breast cancer. (9) Breast cancer patients with positive estrogen and progesterone receptor status (ER+/PR+) have been found to have a greater risk of disease progression and mortality compared to those with negative receptor status (ER-/PR-). These observations are supported by a plethora of studies which highlight the multifaceted nature of breast cancer, with genetic, epigenetic, and environmental factors playing an integral role in the development and progression of the disease. (10,11) This complex interplay underscores the critical need for a comprehensive understanding of these factors in order to identify and address critical modifiable risks, ultimately leading to improved patient outcomes. The tightly regulated process of apoptosis (programmed cell death) serves as a crucial mechanism to maintain cellular homeostasis and prevent the uncontrolled proliferation that underpins cancer. (12) Among the plethora of apoptotic modulators, the Bax gene is a central regulator that exerts its pro-apoptotic effect through interaction with mitochondrial membrane proteins, leading to increased permeability and release of cytochrome C, caspase activation, and ultimately, apoptosis. (13,14) Drug development for breast cancer has been an active field of research, with one notable example being Cisplatin. While Cisplatin is a widely employed chemotherapeutic agent, its utilization in breast cancer treatment is not universal due to its variable efficacy across the diverse molecular subtypes of breast cancer. (15) Nevertheless, its ability to cause DNA cross-linking and trigger DNA damage responses renders it a potential therapeutic avenue for hereditary breast cancers bearing the BRCA1 mutation. (16) This class of breast cancer is susceptible to DNA repair inhibitors, which could potentiate the efficacy of cisplatin and other DNA-damaging agents. (17) Numerous investigations have shed light on the presence of flavonoids in various species of *Centaurea*, a genus of flowering plants with potential medicinal properties. (18, 19) Given the antioxidant capabilities of some plants and their link to cancer prevention, this study aimed to evaluate the differential effects of Cisplatin and *C.behen* extract on Bax gene expression in the MCF-7 breast cancer cell line. This comparison offers valuable insights into the therapeutic potential of *C.behen*, which, if proven effective, could pave the way for novel plant-based cancer therapies.

Materials and Methods

Preparation of *C. behen* extract

C. behen was procured from the Biological Resource Center in Tehran, Iran. The maceration method was employed for extract preparation, wherein 20 grams of plant sample was immersed in 300 milliliters of 80% ethanol solution. The mixture was incubated at 37 ° C for six days, allowing for complete solvent evaporation, and the final extract was stored in aluminum-coated containers until required for experimentation. This rigorous method ensured the consistent and standardized preparation of the *C. behen* extract, essential for reliable evaluation of its therapeutic potential.

Cell culture and MTT assay

The MCF-7 (human breast cancer) cell line was obtained from the Biological Resource Center in Tehran, Iran. The cells were cultured in DMEM growth medium supplemented with a Penicillin-Streptomycin (Pen/Strep) solution of 100 U/ml and 100 µg/ml, respectively, along with 5 ml of fetal bovine serum (FBS). The cell suspension was then incubated in an environment of 5% CO2 at 37°C, providing an optimal environment for proliferation and growth.
The MTT method is based on mitochondrial activity in living cells. This method is based on the reduction and disruption of yellow crystals (tetrazolium) by the enzyme mitochondrial succinate dehydrogenase and the formation of purple crystals. Following the stipulated treatment period, 20 microliters of MTT dye was introduced into the wells containing the cells and extracts. Subsequently, the plate was incubated for 4 hours, enabling the conversion of MTT into the readily measurable purple formazan crystals. After the incubation, 100 microliters of isopropanol reagent was added to the wells and incubated for 15 minutes, which effectively solubilized the formazan product. Finally, the absorbance of the resulting solution was measured using an ELISA reader at a wavelength of 570 nanometers.

Bax expression assay via Quantitative Real-time PCR

To investigate the effect of *C.behen* extract and cisplatin on MCF-7 cells, the cells were incubated with varying concentrations of the extract and cisplatin for a 24-hour and 48-hour treatment period. Total RNA was isolated from each cell sample using the RNX-Plus reagent (Cinnagene® Cat. no.: RN7713C, Iran). The concentration of total RNA was determined using ultraviolet-visible spectrophotometry (ND-1000, Wilmington, DE). The isolated total RNA samples were then reverse-transcribed into complementary DNA (cDNA) using the PrimeScript RT reagent kit (Perfect et al.) RR037A (Takara, Japan), following the manufacturer's guidelines. The synthesized cDNA was then stored at a temperature of -80°C for future use in downstream experiments, ensuring the long-term stability and integrity of the generated cDNA. Real-time polymerase chain reaction (PCR) was conducted with a 20-microliter reaction mixture comprising 10 microliters of RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark), 2 microliters of cDNA at a concentration of 20 nanograms per microliter, 6 microliters of deionized water, 1 microliter of the forward primer, and 1 microliter of the reverse primer at a concentration of 10 picomoles per microliter. The Real-time PCR amplification reaction proceeded as follows: Initially, the reaction mixture was subjected to denaturation at 95°C for 15 minutes to initiate the amplification process. This was followed by a total of 35 cycles comprising three successive temperature transitions: first, 94°C for 15 seconds, allowing for denaturation; next, 60°C for 30 seconds for primer annealing; and finally, 72°C for 30 seconds, which provided the optimal temperature for DNA synthesis. To ensure the accuracy and reliability of the amplification results, the Beta-actin gene was used as an internal control, and each sample was analyzed in duplicate.

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Table 1. Primer sequences used for real-time PCR.

Target genes		Sequences (5`→3`)	Product length
Bax	Forward	GAGCTGCAGAGGATGATTGC	92bp
	Reverse	AAGTTGCCGTCAGAAAACATG	
Beta-actin	Forward	TCCTCCTGAGCGCAAGTAC	89bp
	Reverse	CCTGCTTGCTGATCCACATCT	

Apoptosis Assay

To investigate the apoptotic effect of the *C. behen* extract and cisplatin, on cancerous MCF-7 cell line, an Annexin V-FITC Apoptosis Detection Kit (BioLegend, USA) was employed. The cells (5×10^5) were treated with *C. behen* extract and cisplatin at concentrations equivalent to the IC50 value for 24 and 48 hours. Following the incubation period, the cells were gently trypsinized, washed twice with phosphate-buffered saline (PBS), and resuspended in 100 µL of 1X binding buffer. Subsequently, 5 µL of Annexin V-FITC and 10 µl of Propidium Iodide (50 µg/mL) were added to the reaction tube containing the cell suspension, followed by incubation in the dark at room temperature for 15 minutes. Finally, 400 µL of 1X binding buffer was added to the reaction tube, and Annexin V-FITC binding and Propidium Iodide staining were analyzed using a flow cytometer (BD, USA) with the FITC signal detector (FL1) and phycoerythrin emission signal detector (FL3).

StatisticalAnalysis

To estimate the expression level of the target gene, the 2-△△CT method, also known as the comparative threshold cycle method, was employed using the REST© software tool (Relative Expression Software Tool, Germany). This method involves the normalization of the threshold cycle (CT) values for the target gene against those of the reference gene (Beta-actin in this case) to determine the relative expression of the gene of interest. A P value of less than 0.05 was regarded as statistically significant for all tests conducted, providing an appropriate threshold for establishing statistical confidence in the experimental results.

Results

Assessment of cell viability

To determine the inhibitory effects of *C. behen* extract and cisplatin on the MCF-7 breast cancer cell line, cells were exposed to various concentrations of each treatment agent for 24 and 48 hours, respectively. The 50% inhibitory concentration (IC50) values were then calculated to assess the potency of the treatments. Analysis of the cisplatin-treated cells revealed an IC50 of 2.91 mg/ml after 24 hours of treatment, decreasing to 1.77 mg/ml after 48 hours. Further investigation into the efficacy of *C. behen* extract revealed that the IC50 values for cells treated for 24 hours and 48 hours were 9.64 mg/ml and 7.85 mg/ml, respectively.

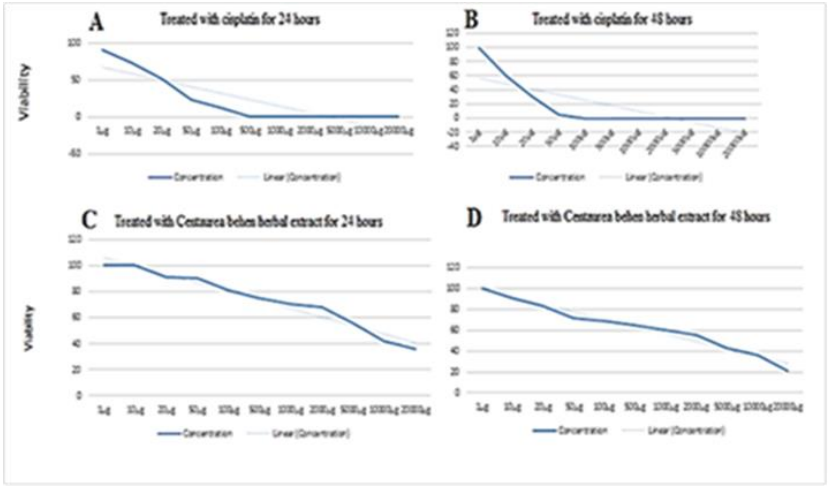

Fig.1. Graph (A) related to treatment with cisplatin for 24 hours, (B) related to treatment with cisplatin for 48 hours, (C) related to treatment *C.behen* extract for 24 hours, and (D) related to treatment *C. behen*extract for 48 hours

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Expression assay of Bax gene under treatment by *C. behen* extract for 24 and 48 hours

The study revealed that there was no significant difference in Bax gene expression between the untreated sample group and the control group (P=0.57), indicating that the baseline expression levels of Bax gene in the MCF-7 cells were comparable between the two groups. Furthermore, the results of the 24 h treatment with *C. behen* extract revealed that the Bax gene expression remained unaltered compared to the baseline level, suggesting that the changes observed in the Bax gene expression after 24 h of treatment were not statistically significant when compared to the control group. After 48 h of treatment with *C. behen* extract, the results revealed a significant increase in the expression level of the Bax gene compared to the baseline expression level in the untreated MCF-7 cells. This increase was observed to be statistically significant when comparing the Bax gene expression in the treated group to the expression in the control group (P=0.009). Figures 2 and 4 provide graphical representations of the Bax gene expression changes in response to *C. behen* extract treatment, illustrating the time-dependent nature of the observed changes.

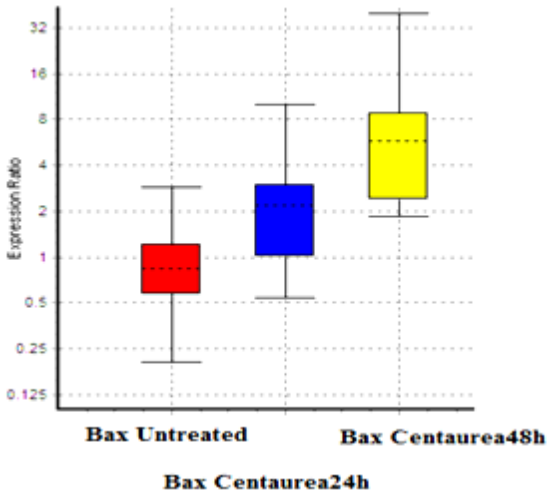

Fig.2. Difference in the level of Bax gene expression under the treatment with *C. behen*

Expression of Bax gene under treatment by Cisplatin for 24 and 48 hours

The results of 24 h cisplatin treatment indicated a significant increase in Bax gene expression relative to the untreated MCF-7 cells, with this increase being statistically significant when comparing the expression levels in the treated group to the control group (P=0.004). When the 48 h cisplatin treatment results were analyzed, a further increase in Bax gene expression was observed, surpassing the expression level after 24 h of treatment. This change in gene expression was statistically significant when compared to the control group (P=0.002), underscoring the potent apoptotic activity of cisplatin on MCF-7 breast cancer cells. Figures 3 and 4 illustrate the dose-dependent increase in Bax gene expression after 24 and 48 hours of cisplatin treatment, respectively, providing a clear visual representation of the gene's activation in response to the anticancer drug.

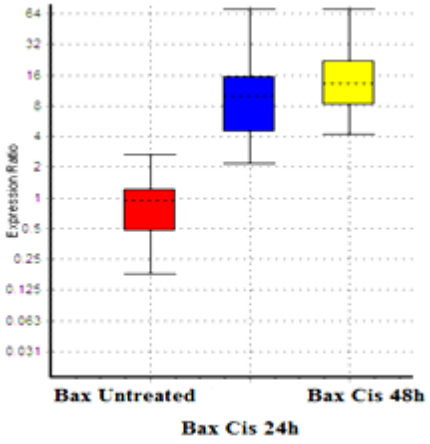

Fig.3. Difference in the level of Bax gene expression under treatment with cisplatin

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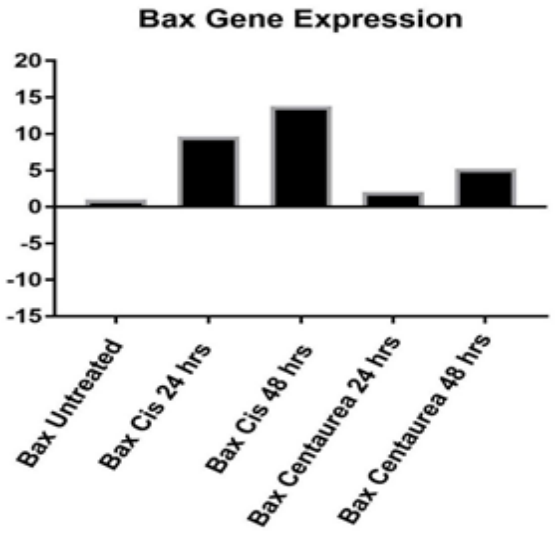
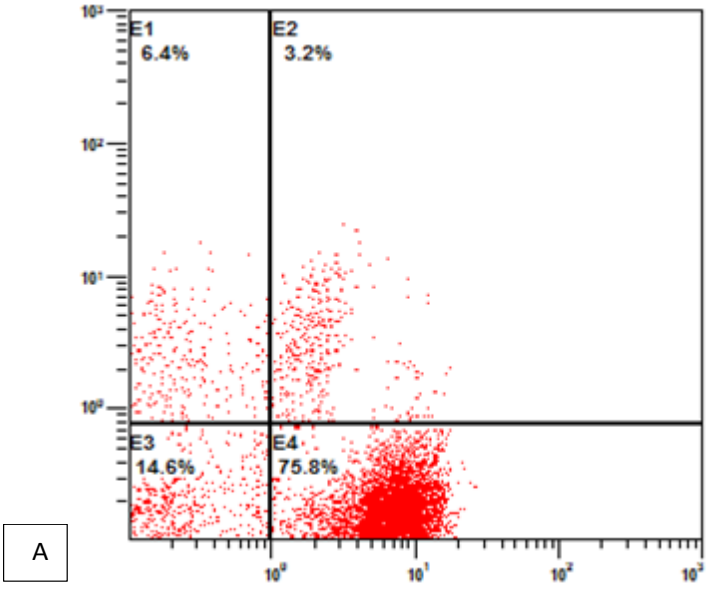


Fig.4. The difference in the level of Bax gene expression under treatment with cisplatin and *C. behen*

Apoptosis Assay

To gain further insights into whether the *C. behen* reduction in the viability of MCF-7 cells related to apoptosis, annexin V and PI staining were conducted. Treatment resulted in the presence of viable (annexin V−/PI−), early apoptotic (annexin V+/PI−), late apoptotic (annexin V+/PI+), and necrotic (annexin V−/PI+) cells. The treatment of MCF-7 cells with both cisplatin and *C. behen* extract resulted in a significant increase in apoptosis when compared to the untreated cells ($P < 0.05$). The extent of apoptosis induced by *C. behen* extract was found to be 40.3% and 52% after 24 and 48 hours of treatment, respectively, while 75.8% and 76.1% of the cells treated with cisplatin underwent apoptosis at the same time points.



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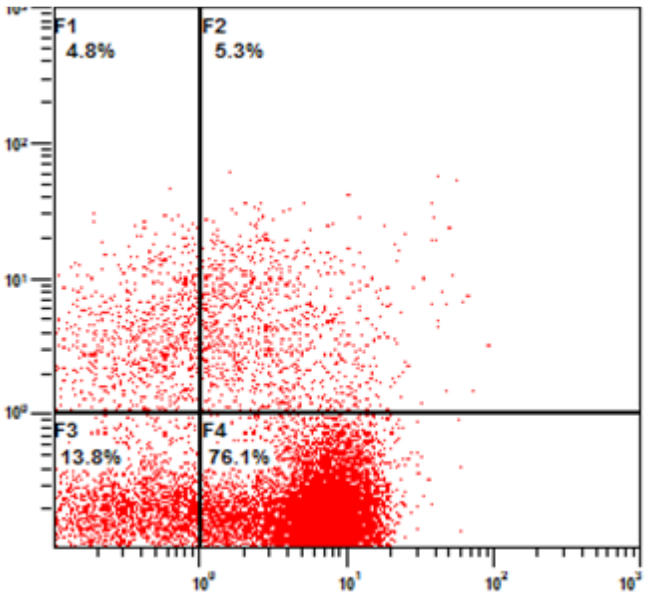

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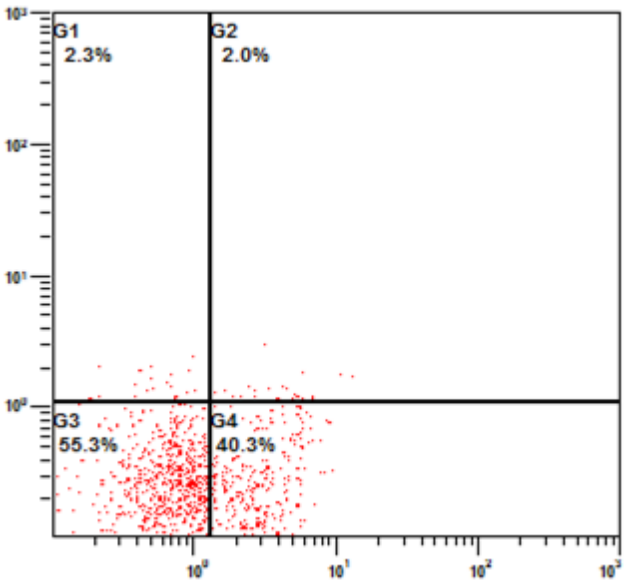

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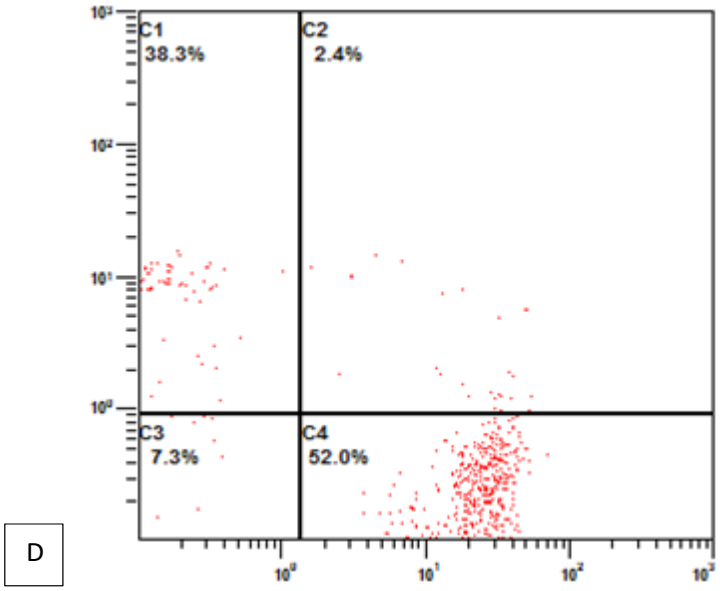

Fig 5. Evaluation of cisplatin and *C. behen* extract on MCF-7 cell line during 24 h and 48 h

A: Cisplatin (24 h) treatment, B: Cisplatin (48 h) treatment C: *C. behen* extract (24 h) D: *C. behen* extract (48 h) treatment

Discussion

Centaurea behen L., also known as Blue Cornflower or Great Star-Thistle, is a perennial member of the Asteraceae or Compositae family native to the eastern Mediterranean region and Iran (20). This hardy plant is widely distributed in these areas and has traditionally been employed in medicinal practices due to its putative therapeutic properties (21). As a resilient species, *C. behen* is able to flourish in diverse environments, particularly temperate climates, making it an adaptable and versatile medicinal plant with potential applications in modern medicine (22). While extensive research on the therapeutic potential of *C. behen* extract for cancer treatment is still ongoing, preliminary findings have demonstrated its anti-proliferative and pro-apoptotic activity against several cancer cell lines, including breast, cervical, and colon cancer (20). Moreover, some preclinical and in vivo studies have demonstrated the extract's efficacy in reducing tumor growth and inhibiting metastasis (23), as well as protecting against chemotherapy-induced side effects (24). The phytochemical composition of *C. behen*, including its antioxidant and bioactive compounds, further supports its potential as a therapeutic agent (25).

In addition, preliminary phytochemical analysis of *C. behen* extract has revealed the presence of various bioactive compounds, such as flavonoids, phenolic acids, and terpenes, which may contribute to its anticancer effects (20,25). These compounds are known for their antioxidant, anti-inflammatory, and pro-apoptotic properties, which are critical in combating cancer progression (26). However, further clinical trials and mechanistic studies are required to fully understand the therapeutic value of *C. behen* extract in cancer treatment, as well as to explore potential synergistic effects with conventional cancer therapies (23,24). Despite these limitations, the promising anticancer properties of this medicinal plant, supported by preclinical evidence, warrant further exploration and development as a potential natural alternative or complementary cancer treatment (27). The gene expression results indicate that *C. behen* extract's effect on **Bax gene expression** in MCF-7 cells is time-dependent. At 24 hours, the expression levels of the Bax gene were comparable between treated and untreated cells, suggesting that the initial cellular response to the extract did not involve significant changes in Bax expression. However, after 48 hours of treatment, a significant upregulation of Bax gene expression was observed in treated cells compared to untreated controls, indicating that *C. behen* extract may activate Bax-mediated apoptotic pathways in breast cancer cells, albeit with a time lag. This delayed upregulation of Bax is consistent with findings from other studies on plant-derived compounds, such as *Curcuma longa* (turmeric) and *Camellia sinensis* (green tea), which also exhibit time-dependent induction of pro-apoptotic genes in cancer cells (28,29). For instance, Khan et al. (2019) reported that green tea polyphenols induced Bax upregulation in MCF-7 cells after 48 hours, similar to the observed effects of *C. behen* extract. Additionally, the time-dependent activation of Bax aligns with the mechanisms of other natural compounds, such as resveratrol and quercetin, which also require extended exposure to initiate apoptosis in cancer cells (30,31). These findings suggest that the delayed apoptotic response may be a common feature of plant-derived anticancer agents, potentially due to the time required for bioactive compounds to accumulate and exert their effects at the molecular level.

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Also, the results indicate that cisplatin rapidly induces Bax gene expression in MCF-7 cells, suggesting that the apoptotic pathway is a primary target of the drug in breast cancer. This increase in Bax expression is dose-dependent and appears to amplify with prolonged exposure to the drug, leading to a greater activation of apoptosis. These findings are consistent with cisplatin's well-established role as a DNA-damaging agent, which triggers apoptosis through mechanisms involving Bax activation and mitochondrial dysfunction.

While these findings provide initial evidence for the potential apoptotic effect of *C. behen* extract on breast cancer cells, further investigations are needed to fully elucidate the mechanism by which the extract upregulates Bax gene expression. It is possible that the extract influences the activity of various regulatory proteins or signaling cascades involved in apoptosis, such as the p53 pathway, or modulates epigenetic factors controlling Bax gene expression (27). For instance, studies on other plant-derived compounds, such as curcumin and resveratrol, have demonstrated their ability to activate p53 and modulate Bax expression through both transcriptional and epigenetic mechanisms (30,31). Additionally, investigations into other apoptotic genes, such as caspases (e.g., caspase-3 and caspase-9), may help to further clarify the molecular basis of the extract's pro-apoptotic effects. For example, similar studies on green tea polyphenols have shown that caspase activation is a critical downstream event in the apoptosis of cancer cells (28).

Overall, these results suggest that both *C. behen* extract and cisplatin may exert anticancer activity through the modulation of Bax gene expression, albeit via different mechanisms and with distinct kinetics. Cisplatin, a well-known chemotherapeutic agent, induces a rapid and potent upregulation of Bax expression, which is consistent with its DNA-damaging properties and direct activation of intrinsic apoptotic pathways (29). In contrast, *C. behen* extract elicits a more delayed but significant upregulation of Bax expression, potentially through epigenetic modifications or indirect signaling pathway modulation (23). This differential modulation of Bax expression highlights the importance of understanding the specific molecular mechanisms underlying each therapeutic agent's apoptotic activity. Such insights could inform the development of optimized treatment strategies, including the potential use of *C. behen* extract as a complementary or alternative therapy to conventional agents like cisplatin, particularly in cases where resistance or toxicity limits the efficacy of standard treatments (24).

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