Scientific Research Publishing

Journal of Biosciences and Medicines, 2025, 13(7), 416-426
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Inhibition of Proliferation and Induction of Apoptosis in Triple-Negative Breast Cancer HCC1937 Cells by Carvacrol

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How to cite this paper: Chen, H.H. (2025) Inhibition of Proliferation and Induction of Apoptosis in Triple-Negative Breast Cancer HCC1937 Cells by Carvacrol. *Journal of Biosciences and Medicines*, **13**, 416-426.
https://doi.org/10.4236/jbm.2025.137033

Received: June 18, 2025
Accepted: July 26, 2025
Published: July 29, 2025

Abstract

Breast cancer is the most commonly diagnosed cancer in women worldwide. Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer with a poor prognosis. Studies have shown the benefits of natural alternatives as an adjuvant breast cancer therapy, including the phenolic monoterpene, carvacrol. However, reports on the antitumor activity of carvacrol against basal-like subtypes of TNBC are scarce. Therefore, the present study investigated the antiproliferative effect of carvacrol on HCC1973 cells and explored the underlying mechanisms. Carvacrol inhibited the proliferation of HCC1973 cells with an IC_{50} of 223.5 μM and induced cell cycle arrest at G0/G1 phase in the treated cells. Furthermore, carvacrol also induced apoptotic cell death in HCC1973 cells as determined by flow cytometry and transmission electron microscopy techniques. Induction of apoptosis was further confirmed at the molecular level using Western blot, where it was shown that carvacrol altered the expression of antiapoptotic and proapoptotic proteins in treated HCC1973 cells. These results suggest that carvacrol could be a potential candidate for basal-like TNBC drug development.

Keywords

Carvacrol, Triple Negative Breast Cancer, Antiproliferative, Cell Cycle, Apoptosis

1. Introduction

In 2020, female breast cancer was the most commonly diagnosed cancer worldwide, surpassing lung cancer, with an estimated 2.3 million new cases [1]. Clinical therapeutic strategies such as surgery, chemotherapy, radiotherapy and hormone

therapy have significantly contributed to the reduction of mortality rate in the last decades. However, recurrence and metastasis are the main reasons for poor prognosis in breast cancer [2]. The triple-negative breast cancer, a subtype of breast cancer, is negative for the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2) and it accounts for approximately 20% of all breast cancer cases [3]. TNBC tends to be more aggressive and highly metastatic with frequent relapse, resulting in poor prognosis outcomes [4]. In 2011, Lehmann *et al.* categorized TNBC into six molecular subtypes, which were later refined to four subtypes: basal cell-like type 1 (BL1), basal cell-like type 2 (BL2), mesenchymal (M) and luminal androgen receptor (LAR) [5]. Each of the subtype display unique biologies. BL1 is one of the most common TNBC subtypes with characteristics of high proliferation rate, elevated cell cycle and DNA damage response [5]. Targeting specific characteristics of each subtype is important for precision treatment of TNBC.

Furthermore, the imbalance between cell proliferation and cell death contributes to tumorigenesis and tumor growth. The killing of cancer cells through programmed cell death or apoptosis remains a goal in clinical cancer therapy [6]. The B-cell lymphoma 2 (Bcl-2) family proteins have proapoptotic (e.g., Bax or Bak) or antiapoptotic (e.g., Bcl-2 or Bcl-xL) activities and they are important in the regulation of apoptotic cell death, tumorigenesis and cellular response to anticancer therapy [6] [7]. Since the discovery of Bcl-2 family proteins, they have become key therapeutic targets for cancer.

Medicinal plants either as extracts or pure compounds have been used all over the world since ancient times [8]. They contain a wide range of bioactive compounds that can treat various diseases. Carvacrol is a natural monoterpene phenol which is widely found in aromatic plants, including thyme, oregano and savory [9]. Besides being used in low concentrations as a food additive, carvacrol is known to possess a variety of biological and pharmacological properties, such as antioxidant, antimicrobial, anti-inflammatory, hepaprotective and vasorelaxant [9]. The compound has also been shown to have anticancer activity against several cancer types, including breast cancer [9] [10], but reports on the anticancer or antitumor activity of carvacrol against basal-like subtypes of TNBC are limited. Therefore, the present study aimed i) to investigate the effect of carvacrol on the proliferation of TNBC BL1 subtype, HCC1973 cells and ii) to elucidate the underlying mechanism involved in the induction of apoptosis in the carvacrol-treated HCC1973 cells.

2. Materials and Methods

Materials

The RPMI-1640 medium and Protein Extraction Kit were purchased from American Image (Wuhan) Technology Co., Ltd. 3-2(4,5-Dimethylthiazole-2)-2-tetramethylazothiazole blue (MTT), the propidium iodide (PI) dye, and the Annexin V-FITC/PI apoptosis detection kit were purchased from Shanghai Jingke

Chemical Technology Co., Ltd. The rabbit anti-human Bcl-2, anti-human CytC, anti-human Caspase-3, anti-human β-actin polyclonal antibodies, and the goat anti-rabbit HRP secondary antibody were purchased from Wuhan Yipu Biotechnology Co., Ltd.

Cell line and cell culture

HCC1937 human breast cancer cells were purchased from the Beijing Beina Chuanglian Biotechnology Research Institute. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 IU/ml penicillin/streptomycin. The cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

MTT assay

HCC1937 cells were seeded in 96-well plates at a density of 1×10^4 cells/well. After 24 h incubation, cells were treated with increasing concentrations of carvacrol (0 - 80 μM) for 24 and 48 h. Then, 20 μL of 5 mg/mL MTT was added to the wells and incubated for 4 h at 37˚C. After removing the medium and dye, 200 μL DMSO was added to the wells to dissolve the formazan. The absorbance was measured using a microplate reader at 570 nm. The inhibition of cell proliferation was calculated using the following equation:

$$\text{Cell proliferation inhibition rate } (\%) = \frac{(\text{OD of control} - \text{OD of sample})}{\text{OD of control}} \times 100$$

Cell cycle analysis

After treatment for 24 h with 0 - 80 μM carvacrol, HCC1937 cells were trypsinized and collected for centrifugation (1000 rpm for 10 min). Then, the cells were resuspended in phosphate-buffered saline (PBS). Pre-chilled 70% absolute ethanol was added to fix the cells at 4˚C. Subsequently, the mixture was centrifuged, and the supernatant was discarded. After washing, the cell pellet was resuspended in PBS and 50 μg/mL PI dye was added. After 10 min, the cell cycle distribution was detected using a flow cytometer (CytoFLEX; Beckman Coulter Life Sciences, USA). The percentages of cells in the G_1 phase, S phase and G_2/M phase for each treatment were calculated.

Apoptosis assay

HCC1937 cells treated with 0 - 80 μM carvacrol for 24 h were washed with PBS, trypsinized and centrifuged at 1000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended with fresh media to obtain a cell suspension, which was adjusted to a concentration of 1×10^6 cells/mL. Then, 100 μL of the cell suspension was mixed with 5 μL Annexin V-FITC and 10 μL of 20 μg/mL PI and incubated for 30 min. Apoptotic cells were detected using flow cytometry. Percentage of apoptosis rate was calculated using the following equation:

$$\text{Apoptosis rate } (\%) = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells}} \times 100$$

Transmission electron microscopy (TEM)

Following treatment with carvacrol for 24 h, HCC1937 were trypsinized and centrifuged for 10 min at 1000 rpm. The supernatant was discarded and 0.25%

glutaraldehyde was added slowly along the centrifuge tube wall. After ethanol dehydration, the cells were embedded in paraffin and sectioned. The sections were double stained with uranyl acetate and lead citrate, and the changes in the internal structure of the cells were observed using a transmission electron microscope (HDMI3800-A; Shenzhen Seepack Optical Instrument Co., Ltd., Shenzhen, China).

Western blot analysis

Following treatment with increasing concentrations (0 - 80 μM) of carvacrol for 24 h, HCC1937 cells were lysed with RIPA solution on ice and incubated for 20 min. The mixture was centrifuged at 3000 rpm for 10 min and the supernatant was obtained to detect the protein levels. The protein levels were quantified using the Bradford assay. Then, 20 μg of protein was mixed with an equal amount of loading buffer. The proteins were denatured in a boiling water bath, separated using electrophoresis, and transferred onto a membrane. The membrane was blocked with 5% skimmed milk powder for 2 h and then washed with TBST. This was followed by overnight incubation with antibodies against Bax, CytC, Caspase-3 and the internal control β-actin (1:500) at 4˚C. After washing with TBST, the HRP-conjugated goat anti-rabbit secondary antibody (1:1000) was added and incubated at 37˚C for 1.5 h. Protein bands were developed and the relative expression of each protein was calculated.

Statistical analysis

All data are represented as means ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, USA). Student's t-test was used to compare the mean of each group. A p-value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

Inhibition of proliferation of HCC1937 cells by carvacrol

The antiproliferative activity of carvacrol against HCC1937 cells was assessed using MTT assay. As shown in **Figure 1**, treatment with 60 and 80 μM carvacrol significantly inhibited the proliferation of HCC1937 cells, with the later having more than two-fold higher activity than that of control. Carvacrol significantly inhibits the cell growth of HCC1937 cells in a dose and time-dependent manner. The IC_{50} values of carvacrol at 24 and 48 h were 320 and 223.5 μM, respectively.

Induction of cell cycle arrest in HCC1937 cells by carvacrol

To further investigate the inhibitory effect of carvacrol on HCC1937 cells, cell cycle distribution in treated cells was analyzed by flow cytometry. Treatment with carvacrol showed a substantial increase in cell proportion at G0/G1 phase compared to control (**Figure 2**). Besides, results also showed that the porpotion of HCC1937 cells at the G0/G1 phase significantly increased with increasing concentration of carvacrol, suggesting that the effect was concentration-dependent (**Figure 2(B)**). Conversely, a decrease in the proportion of cells at the S and G2/M

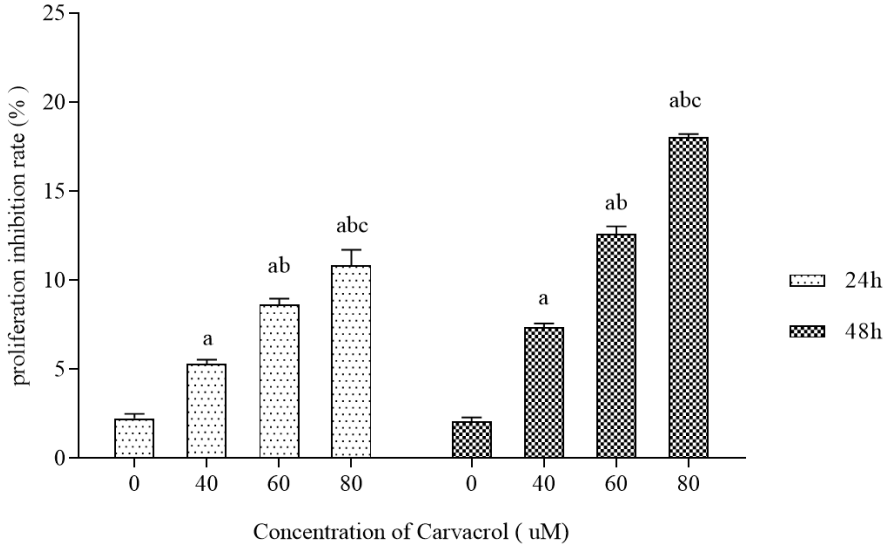

Figure 1. Inhibitory effect of carvacrol on HCC1937 cells. Data represents mean ± SD from three independent experiments (n = 3). [a]$p < 0.05$ vs control; [b]$p < 0.05$ vs 40 μM carvacrol; [c]$p < 0.05$ vs 60 μM carvacrol.

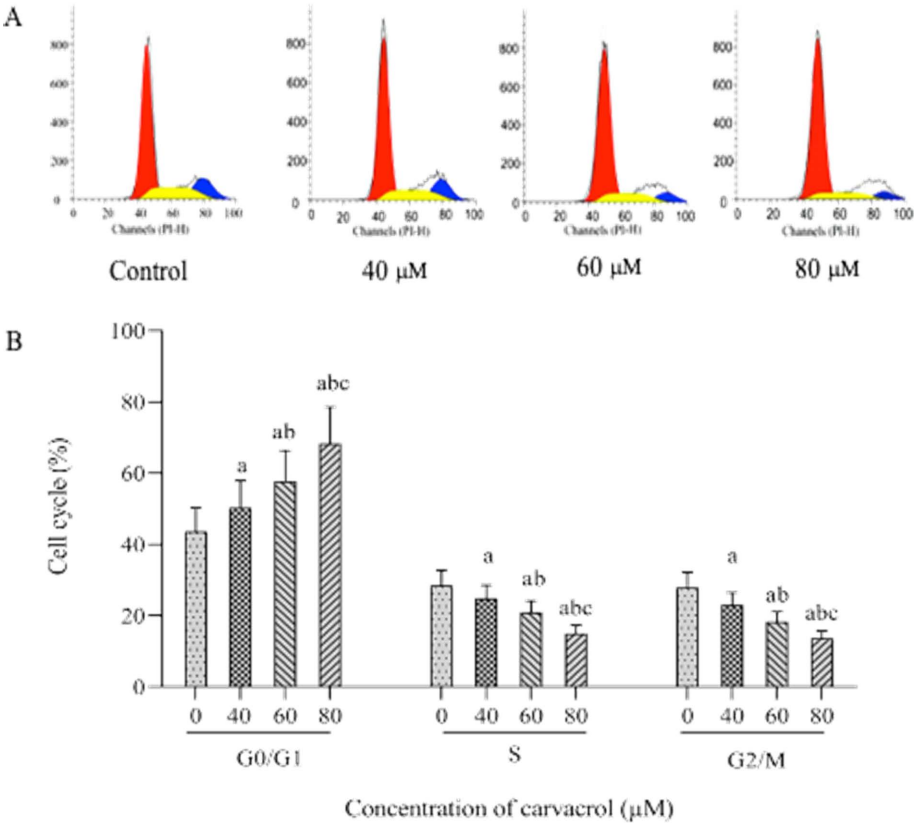

Figure 2. Induction of cell cycle arrest in HCC1937 cells by carvacrol. Cell cycle distribution of carvacrol-treated HCC1937 cells determined by flow cytometry (A). The peaks correspond to the G0/G1 (red), S (yellow) and G2/M (blue) phases. The percentage of cells in each phase of the cell cycle (B). Data represents mean ± SD from three independent experiments (n = 3). [a]$P < 0.05$ vs control; [b]$p < 0.05$ vs 40 μM carvacrol; [c]$p < 0.05$ vs 60 μM carvacrol.

phases was observed. The findings suggest that carvacrol induced cell cycle arrest at G0/G1 phase, which could be responsible for the antiproliferation of treated HCC1937 cells.

Induction of apoptosis in HCC1937 cells by carvacrol

Apoptosis in HCC1937 cells induced by carvacrol was assessed by flow cytometric Annexin V/PI assay. Treatment with carvacrol showed a significant increase in the proportion of apoptotic cells. The total percentages of cells undergoing early and late apoptosis on exposure to 40, 60 and 80 μM for 24 h were 20.4%, 31.6% and 53.4%, respectively (**Figure 3(A)**). Furthermore, the apoptosis rate in cells treated with the highest concentration of carvacrol was 4-times higher than that of control (**Figure 3(B)**).

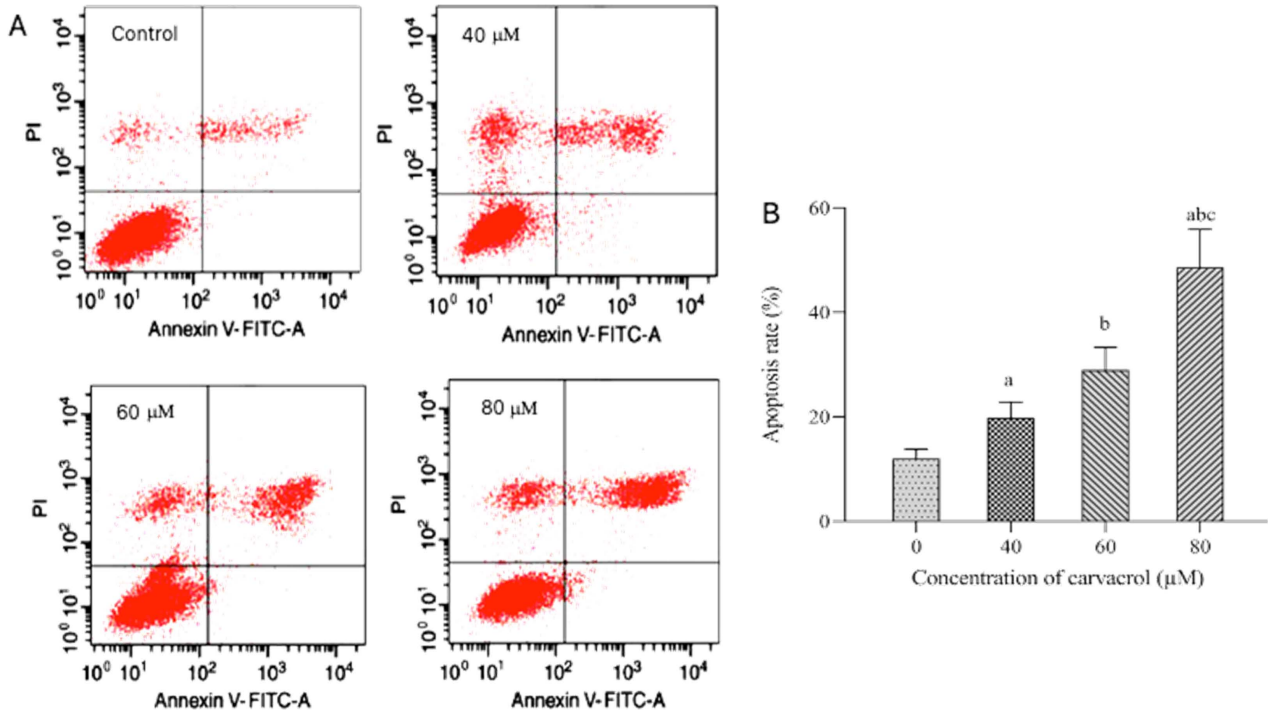

Figure 3. Flow cytometric analysis of HCC1937 cells treated with increasing concentrations of carvacrol for 24 h (A). Relative apoptosis rate of cells exposed to 0 - 80 μM carvacrol. Data represents mean ± SD from three independent experiments (n = 3). [a]$p < 0.05$ vs control; [b]$p < 0.05$ vs 40 μM carvacrol; [c]$p < 0.05$ vs 60 μM carvacrol.

Induction of ultrastructural changes in HCC1937 cells by carvacrol

The ultrastructural changes in carvacrol-treated and untreated (control) HCC1937 cells were observed under a transmission electron microscope. The untreated HCC1937 cells had large, clear nuclei, rich in organelles and matrix, and evenly distributed nuclear chromatin (**Figure 4(A)**). In contrast, the treated HCC1937 cells had lost their microvilli and their cytoplasm had degenerated (**Figures 4(B)-(D)**). Moreover, chromatin condensation, which is an important morphological hallmark of apoptosis was also seen in the treated cells.

Modulation of apoptosis-related proteins in HCC1937 cells by carvacrol

The underlying mechanism involved in the induction of apoptosis in HCC1937

cells by carvacrol was elucidated by Western blot analysis through examining the expression of apoptosis-related proteins (**Figure 5(A)**). The expression of anti-apoptotic protein, Bcl-2 was significantly reduced in a concentration-dependent manner (**Figure 5(B)**). Meanwhile, the pro-apoptotic protein, Bax was activated

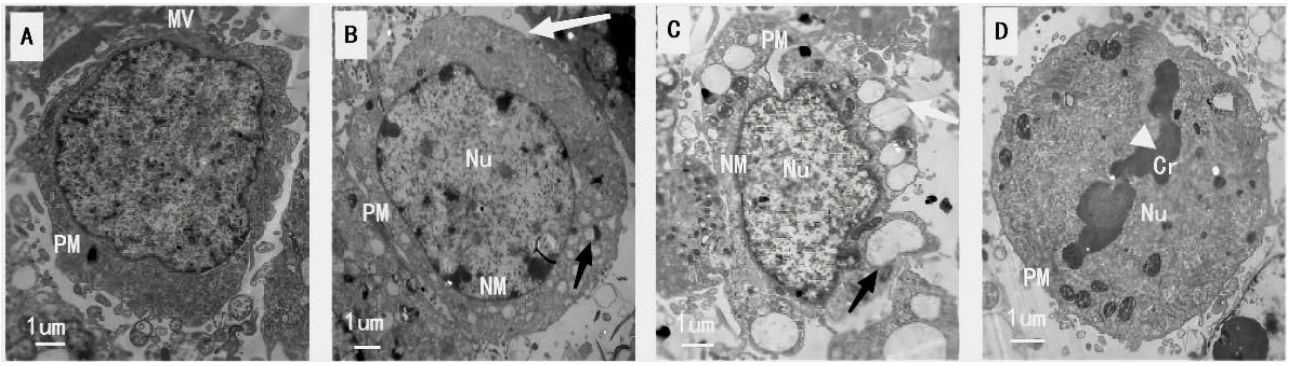

Figure 4. Transmission electron micrographs (4000×) of the control (untreated) HCC1937 cell (A) and cells treated with the carvacrol at 40 μM (B), 60 μM (C) and 80 μM (D) for 24 h. Distinct morphological changes, including plasma membrane alteration (white arrow), chromatin condensation (white arrowhead), were observed in the treated cells. Cr: chromatin, MV: microvilli, NM: nuclear membrane, Nu: nucleus, PM: plasma membrane.

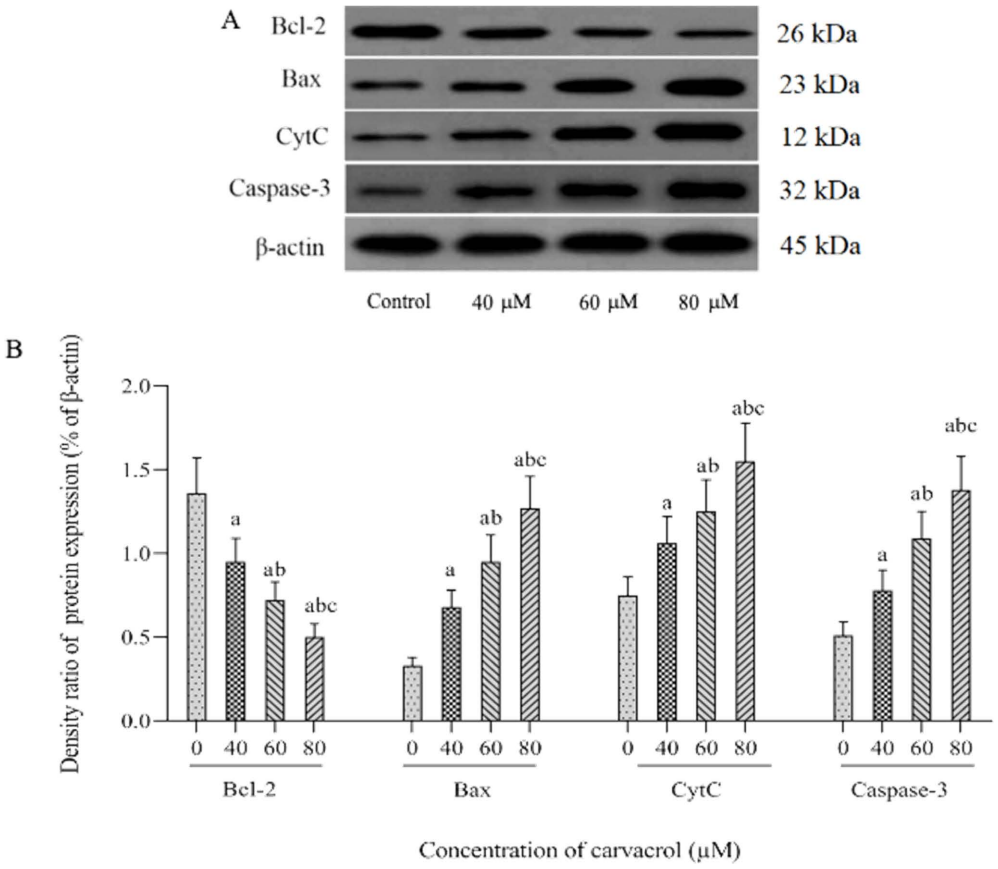

Figure 5. Western blot analysis demonstrating the induction of apoptosis in carvacrol-treated HCC1937 cells. The expression of apoptosis-related proteins (Bcl-2, Bax, CytC and Caspase-3) following exposure to different concentrations of carvacrol for 24 h (A). Relative density ratio of the studied proteins to β-actin (B). Data represents mean ± SD from three independent experiments (n = 3). [a]$p < 0.05$ vs control; [b]$p < 0.05$ vs 40 μM carvacrol; [c]$p < 0.05$ vs 60 μM carvacrol.

following treatment with carvacrol and the protein considerably increased with increasing concentrations of carvacrol. Treated cells also showed an increase in the expression of cytochrome c, which resulted in the increased expression of caspase-3.

4. Discussion

Female breast cancer is currently the most common type of cancer worldwide [1]. Continuous improvement of treatment modalities is therefore necessary. Carvacrol has been reported to exhibit tumor inhibitory and cytotoxic effects on HCT116 human colon cancer cells and A549 lung carcinoma epithelial cells, respectively [11]. Moreover, Li *et al.* (2021) also showed that carvacrol reduced the viability of different types of breast cancer cells, including BT-483, BT-474, MCF-7, MDA-MB-231, and MDA-MB-453 [12]. However, the antiproliferative and apoptosis mechanisms induced by carvacrol in HCC1937 cells are unclear. In the present study, the tetrazolium dye-based assay revealed significant inhibition of proliferation of HCC1937 cells by carvacrol in a concentration-dependent manner. Further investigation on carvacrol's inhibitory effect by flow cytometry showed that the compound was able to induce cell cycle arrest at G0/G1 phase in HCC1937 cells, inhibiting DNA synthesis at the S phase. This suggests that the induction of cell cycle arrest may contribute to the antiproliferative potential of carvacrol. The results from this study are in agreement with previous studies that have shown the ability of carvacrol to induce cell cycle arrest at G0/G1 phase in other types of breast cancer cells, including MDA-MB-231 [12] and MCF-7 [13]. HCC1937 cell line belongs to the BL1 subtype of TNBC [14], which is characterized by increased cell proliferation, loss of cell cycle control and abnormal expression of DNA damage response genes [15]. Therefore, inhibiting cell proliferation and DNA damage response as well as retarding the cell cycle may be promising therapeutic strategies for the treatment of BL1 TNBC [14] [16].

Induction of cell cycle arrest at G0/G1 phase will subsequently lead to apoptosis [17]. Apoptosis is a physiological process of programed cell death, characterized by distinct morphological features, including shrinkage of cell, formation of apoptotic bodies and condensation of nuclear chromatin [18]. Generally, there are two main apoptotic pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway. Each pathway requires specific signaling molecules to initiate a molecular event. For instance, the intrinsic pathway involves the Bcl-2 family of proteins, where some of them are pro-apoptotic (e.g., Bax, Bak, Bid, Bad) and some are anti-apoptotic (e.g., Bcl-2, Bcl-XL). The Bcl-2 proteins regulate the release of cytochrome *c* from the mitochondria by altering the permeability of the mitochondrial membrane. Subsequently, the initiator caspase-9 will be activated in the intrinsic pathway, which in turn will activate the executioner caspase-3, leading to apoptosis. Meanwhile, the extrinsic pathway involves transmembrane receptor-mediated interactions that are engaged by major proteins, such as TNF-*α*, TNFR1, FasL, FADD, TRAIL and DR3. These proteins will activate the initiator

caspase-8 in the extrinsic pathway and eventually caspase-3 [18].

In this study, Annexin V/PI staining was used to assess the cell death in HCC1937 cells induced by carvacrol. During early apoptosis, the asymmetric distribution of phospholipids is lost and the phosphatidylserine (PS) is translocated from the inner to the outer plasma membrane leaflet. The exposed PS interacts strongly and specifically with Annexin V and hence, the dye is used to detect early apoptosis [19]. On the other hand, the loss of membrane integrity in late apoptotic and dead cells results in the ability of PI to enter and stain the cells [20]. Flow cytometric analysis in this study revealed that carvacrol-treated HCC1937 cells significantly underwent apoptosis compared to untreated cells and the apoptosis rate increased in a concentration-dependent manner. Moreover, TEM analysis also confirmed the induction of apoptosis by carvacrol, where treated HCC1937 cells displayed morphological characteristics of apoptotic cell death, including nuclear condensation. To further clarify the mechanism involved in the induced cell death, expression of apoptosis-related proteins was examined using Western blot. In response to cellular stress, Bax induces apoptosis by the loss of mitochondrial permeability. Conversely, Bcl-2 inhibits the effect of Bax by forming a heterodimer Bcl-2/Bax, which prevents the formation of Bax/Bax homologous dimer essential for the early step of apoptosis [21]. In the carvacrol-treated HCC1937 cells, there was a significant decrease in Bcl-2 expression level, accompanied by a considerable increase in Bax expression and subsequently an increased level of cytochrome *c* expression, leading to apoptosis through activation of caspase-3. This demonstrates the therapeutic potential of carvacrol in inducing apoptosis in HCC1937 cells via the mitochondrial-mediated intrinsic pathway by modulating the Bcl-2 family proteins. Several studies have also reported the initiation of apoptosis by carvacrol through the intrinsic pathway, such as in MCF-7 [22], MDA-MB-231 and human non-small cell lung cancer (NSCLC) cell lines [9].

5. Conclusion

The present study demonstrated the antiproliferative and apoptotic activities of carvacrol against the BL1 TNBC cell line, HCC1937, using colorimetric, flow cytometric, transmission electron microscopy and Western blot techniques. Carvacrol arrested the cell cycle of treated cells at G0/G1 phase and induced apoptotic cell death via the intrinsic apoptosis pathway by altering the expression levels of Bcl-2, Bax, cytochrome *c* and caspase-3 proteins. These observations suggest that carvacrol could be a potential chemotherapeutic agent against BL1 subtype of TNBC.

Statements and Declarations

Data availability

Data are available on request from the corresponding author. This article was deposited as a preprint onto Research Square (DOI: https://doi.org/10.21203/rs.3.rs-2105532/v1).

Acknowledgements

This study was supported by the Gansu Province Higher Education Innovation Fund project (grant nos. [2025A-210]), Qingyang Science and Technology Planning Project (grant nos. [QY-STK-2024B-178]) and Industry-University-Research Co-operation Projects (grant nos. [HXZK2472]).

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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