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# Carvacrol Induces Doxorubicin-Resistant Breast Cancer Cell Apoptosis via Inhibition of the PI3K/Akt Pathway

**Hu-Hu Chen[1,2]*, Jiao-han Zhou[3] and Siat Yee Fong[2,4]**

[1]*Medical College, Longdong University, Gansu 745700, China*
[2]*Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, Jalan UMS, Kota Kinabalu, Sabah 88400, Malaysia*
[3]*Department of Ultrasound Medicine, The Second People's Hospital of Qingyang City, Gansu 745700, China*
[4]*Borneo Medical and Health Research Centre, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, Jalan UMS, Kota Kinabalu, Sabah 88400, Malaysia*

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**HHC, JHZ and SYF conceived and designed the experiments. HHC performed the experiments and wrote the manuscript. JHZ and SYF analyzed the data. All authors have read and approved the final version of the manuscript.**

**Key words**
**Carvacrol, Breast cancer, Apoptosis, Dox-resistance, PI3K/Akt signaling pathway**

## ABSTRACT

Doxorubicin (Dox), a widely used chemotherapeutic agent for breast cancer, is often ineffective due to the development of Dox resistance, posing a significant challenge in cancer treatment. Carvacrol is a monoterpene phenol with diverse biological activities, has emerged as a potential anticancer agent. However, its antitumor effects and underlying mechanism against Dox-resistant breast cancer remain largely unexplored. This study investigated the effect of carvacrol on cell viability, cell cycle progression, and apoptosis in Dox-resistant triple negative breast cancer (TNBC) MDA-MB-231/Dox cells. Western blotting analyses were performed to access protein levels associated with apoptosis and the PI3K/Akt pathway. The results revealed that carvacrol significantly inhibited MDA-MB-231/Dox cell proliferation in a concentration-dependent manner compared to the control ($p$ <0.05). Furthermore, carvacrol treatment increased the distribution of MDA-MB-231/Dox cells in the $G_0/G_1$ phase while decreasing the distribution in the S and $G_2$/M phases ($p$ <0.05). Moreover, carvacrol treatment enhanced the apoptosis rate and Bax protein expression, while decreasing Bcl-2, PI3K and P-Akt protein levels, indicating that carvacrol treatment induced cell apoptosis by deactivating the PI3K/Akt signaling pathway. These findings suggest that carvacrol holds promise as a therapeutic strategy for Dox-resistant breast cancer, providing novel insights into the development of effective therapies that address drug resistance and improve patient outcomes.



## INTRODUCTION

Breast cancer remains one of the leading causes of cancer-related deaths among women worldwide. In 2020, breast cancer accounted for over 680,000 female deaths, representing approximately 15.5% of all female cancer fatalities (Sung *et al*., 2021). Triple-negative breast cancer (TNBC) is a particularly aggressive subtype, characterized by the absence of estrogen, progesterone, and HER2 receptors. It is the most refractory to current anticancer therapies and is associated with a high degree of aggressiveness and metastatic development, with very frequent relapses and a poor prognosis. MDA-MB-231 is a highly aggressive and metastatic cell line that undergo the epithelial-to-mesenchymal transition (EMT) process, which is linked to cancer progression (Cailleau *et al*., 1978). Doxorubicin (Dox) is one of the potent chemotherapy options commonly used in first- or second-line chemotherapy regimens for localized or metastatic breast cancer (Lankelma *et al*., 1999). However, the emergence of Dox resistance has proven to be a major limitation for clinical chemotherapy failure (Chen *et al*., 2018a; Lovitt *et al*., 2018). While bevacizumab has been used in some countries to treat TNBC when combined with chemotherapy (Brufsky *et al*., 2011), its clinical benefits remain limited and has not shown to significantly improve survival (Collignon *et al*., 2016).

Increasing evidence suggests that tumor resistance

* Corresponding author: chenhongfei202106@163.com
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stems not only from drug distribution and accumulation in cells, but also from imbalanced and dysregulated signaling pathways that regulate cell proliferation and apoptosis in cancer progression (Liu *et al*., 2014; Pommier *et al*., 2004). The PI3K/Akt signaling pathway plays a crucial role in regulating chemically induced apoptosis, and its aberrant activation is associated with cancer progression (Cui *et al*., 2022; Noorolyai *et al*., 2019; Vara *et al*., 2004). This signaling pathway is central to the regulation of cell proliferation, growth, metabolism and motility in cancer (Martini *et al*., 2014; Song *et al*., 2019). Given its inhibition leads to a pronounced anticancer activity, some inhibitors of this pathway for cancer treatment have been approved and applied (Li *et al*., 2016). PI3K (phosphatidylinositol 3-kinase) and Akt (protein kinase B) are the two primary proteins components of the PI3K/Akt signaling pathway. PI3k, a member of the lipid kinase family, mediates extracellular signal transduction and promotes cell growth. Activation of PI3K can affect cell growth, proliferation and survival, as well as cycle progression and apoptosis. AKT is a crucial protein that play significant roles in regulating cell growth, survival, proliferation, apoptosis, and glycogen metabolism (Song *et al*., 2019). This signaling pathway serves as a link between HER2 signaling and the transcription of genes regulated by Era, and is associated with MDR1 upregulation and chemotherapeutic resistance (Nahta and O'Regan, 2012).

Medicinal plants have long been used worldwide for their therapeutic potentia, either as extracts or pure compounds. These plants contain a plethora of bioactive compounds that have shown utility in treating a variety of diseases. Carvacrol is a natural monoterpene found in various aromatic plants, such as thyme, savory, and oregano. Carvacrol has gained recognition for its diverse pharmacological properties, including antioxidation, anti-microbiosis, anti-inflammation, hepatic protection and vascular relaxation effects (Suntres *et al*., 2015). Carvacrol has also exhibited anti-cancer activity against a range of different cancer types, including breast cancer (Sharifi-Rad *et al*., 2018).

Nonetheless, carvacrol's antitumor activity or anticancer mechanism against MDA-MB-231/Dox cells (Dox-resistant TNBC) is relatively unexplored. Therefore, this study aimed to evaluate how carvacrol affects MDA-MB-231/Dox cells *in vitro*.

## MATERIALS AND METHODS

*Cell culture and development of drug resistant cells*

MDA-MB-231 cell line was procured from Yimo Biotechnology Co., LTD. (Xiamen, China), and cultured in DMEM supplemented with 4500 mg/L glucose, 10% FBS and 1% penicillin–streptomycin. Cells were maintained in 5% $CO_2$ at 37°C and the growth medium was refreshed every 48 h. Upon reaching 80-90% confluence, cells were subjected to trypsinization and subculturing, Doxorubicin was purchased from Aladdin Biochemical Technology Co., Ltd., Shanghai, China, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Life Technologies, Carlsbad, CA, USA, Carvacrol, trypsin and propidium iodide (PI) were purchased from Sigma-Aldrich, St. Louis, MO, USA, Primary antibodies against Bcl-2, Bax was purchased from Elabscience Biotechnology Co., Ltd., Wuhan, China, Primary antibodies against PI3K, p-Akt, GAPDH and secondary antibody was purchased from Cell Signaling Technology, Danvers, Ma, USA, Annexin V-FITC/PI double staining kit, Caspase-3/CPP32 colourimetric assay kit, and Cell Counting Kit-8 was purchased from eBioscience Inc., San Diego, CA, USA. RIPA buffer was product of Thermo Fisher Scientific, Waltham, MA, USA.

The Dox-resistant MDA-MB-231 cell subline (MDA-MB-231/Dox) was established following existing protocols (Han *et al*., 2019). Briefly, MDA-MB-231 cells were cultured in medium containing 0.24 μM, equivalent to 1/10 of the 50% inhibitory concentration ($IC_{50}$). The drug concentration was subsequently increased by 1.5-fold at each resistance level, reaching a maximum concentration of 6.2 μM. At each level, cells were cultured for at least 4 weeks, with medium replaced every 3 days. Finally, the established MDA-MB-231/Dox cells were trypsinized and subcultured at 80-90% confluence. After at least four weeks of cultivation in drug-free medium, subsequent experiments were performed.

*Cell proliferation measurement*

Cell proliferation was accessed using the Cell Counting Kit-8 (CCK-8) assay. Briefly, a total of 5000 MDA-MB-231/Dox and parental cells per well were added to a 96-well microplate (100 μL/well) and cultured for 24 h. Subsequently, cells were subjected to varying concentrations of Dox (0–60 μM) for 24 h. The absorbance at 450 nm was measured, the resistance index (RI) was calculated based on the IC50 value of the drug-resistant cells relative to that of the parental cells.

To determine the prolonged stability of MDA-MB-231/Dox cells, cells were cultured for 3 months without Dox exposure, and their sensitivity to Dox was assessed every 3 weeks. The impact of carvacrol on MDA-MB-231/Dox cell viability was determined using the CCK-8 assay following treatment with various concentrations of carvacrol for defined periods.

*Cell cycle evaluation*

The influence of carvacrol on the MDA-MB-231/

Dox cell cycle was evaluated using a PI (propidium iodide) staining assay. Specifically, T25 flasks were used to incubate cells ($1 \times 10^5$) for 24 h. Cells were exposed to carvacrol at varying concentrations (0–200 μM) at 37°C for 24 h. Cells were then fixed with cold ethanol (70%), rinsed with PBS, and resuspended in a PI and RNase A-enriched staining solution for 15 min. After incubation, cells were analyzed by flow cytometry (BD Bioscience, Bedford, MA, USA).

*Cell apoptosis assay*

MDA-MB-231/Dox cell line was exposed to carvacrol at various concentrations (0, 100, 150, and 200 μM) for 24 h. Cell apoptosis was assessed through flow cytometry using an Annexin V-FITC/PI double staining kit (Rinaldi *et al*., 2021).

Caspase-3/CPP32 colorimetric assay kit was utilized to determine the caspase-3 activity, and was measured through comparison with Dox alone and calculated using the following formula:

$$\text{Caspase activity (\%)} = \frac{OD_{405} \text{ of sample}}{OD_{405} \text{ of control}} \times 100$$

At least three replicate wells were used for each drug concentration, and the testing was carried out 3 times independently.

*Western blotting*

Approximately $1 \times 10^4$ MDA-MB-231/Dox cells were treated with carvacrol (0, 100, 150, 200 μM) in T25 flasks for 48 h. Following cell lysis utilizing RIPA buffer and protein quantification through Bradford assay, 50 μg of protein sample was resolved by SDS–PAGE, transferred onto nitrocellulose membranes, subsequently probed with specific antibodies at 4°C overnight. This was followed washed 5 times with 0.1% PBS-Tween and incubated with the appropriate secondary antibody for 3 h. They were then washed five times, and images were quantified using Image Quant software (GE Healthcare, Piscataway, NJ, USA).

*Statistical analysis*

The data were presented as the mean Mean ± SD (standard deviation). Comparisons were conducted using Student's *t*-test or one-way ANOVA ($p < 0.05$ indicating statistical significance).

## RESULTS

*The effect of carvacrol on the proliferation of Dox-resistant MDA-MB-231 cells*

The CCK-8 assay revealed a dose-dependent inhibition in both MDA-MB-231/Dox and parent cell lines after 24 h exposure to Dox (0–60 μM) (Fig. 1A). Notably, MDA-MB-231/Dox cells exhibited higher resistance to Dox than parental cells, with IC50 values of 10.54 ± 0.24 μM and 2.38 ± 0.15 μM, respectively ($p$ <0.01, RI = 4.43). The IC50 values for Dox following 3 months without exposure to Dox were 2.37 ± 0.12 μM and 10.51 ± 0.23 μM for parental and MDA-MB-231/Dox cells, respectively, while there was no significant difference between their original Dox IC50 values, thus verifying the successful establishment of a Dox-resistant TNBC cell line.

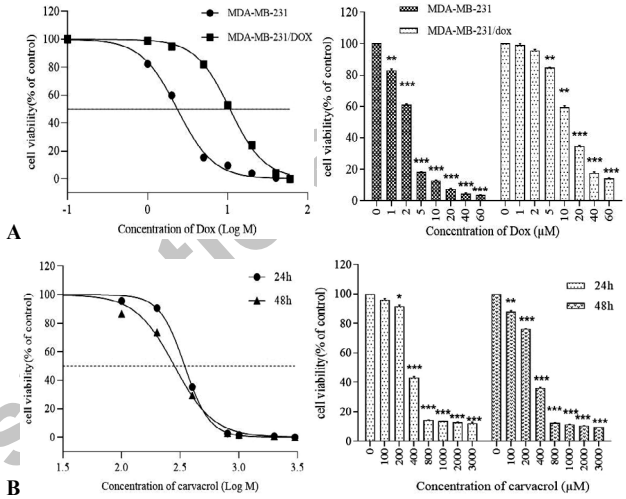


Fig. 1. (A) Dose response curve of MDA-MB-231 and MDA-MB-231/Dox cells to Dox. (B) Carvacrol inhibits the proliferation of MDA-MB-231/Dox cells as measured by CCK-8 assay. *P*-values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ are indicated with (*), (**), and (***) vs. black control, respectively. Plotted values are means ± SD (n = 3), each one in triplicate.

Carvacrol treatment (0–3000 μM) for 24 and 48 h resulted in a dose- and time-dependent inhibition of MDA-MB-231/Dox cell proliferation (Fig. 1B). The IC50 values of carvacrol were 344.7 ± 5.7 μM at 24 h and 281.8 ± 5.3 μM at 48 h. After a 24-h exposure, carvacrol concentrations below 200 μM had little effect on cell viability, with no observable cell death. Hence, 100, 150, and 200 μM carvacrol were preferred for subsequent experiments to better evaluate their impact on MDA-MB-231/Dox cell growth.

*Carvacrol induced G0/G1 cell accumulation in MDA-MB-231/Dox cells*

Following a 24-h exposure to carvacrol at various concentrations, flow cytometry was performed to characterize the cell cycle profiles in MDA-MB-231/Dox cells. Noticeably, MDA-MB-231/Dox cells in G0/G1 phase were more abundant in groups treated with carvacrol

at 100, 150, and 200 μM than in the untreated control group. These results imply that carvacrol can induce cell cycle arrest in G0/G1 phase, hinder DNA synthesis in S phase and promote apoptosis in these cells, as detailed in Figure 2.

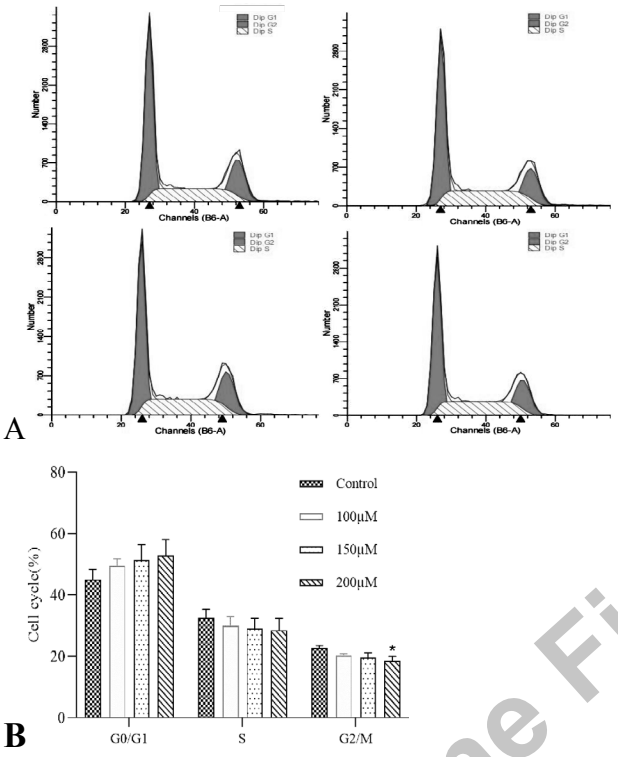


Fig. 2. Cell cycle distribution of MDA-MB-231/Dox cells after exposing to various concentrations of carvacrol for 24 h. (A) Representative flow cytometric histogram of cell cycle progression. (B) Percentage of total number of cells. Notes: Values are presented as mean ± SD. * $p < 0.05$, compared to untreated control.

*Carvacrol induced apoptosis in MDA-MB-231/Dox cells*

Upon treating MDA-MB-231/Dox cells with carvacrol (0, 100, 150, 200 μM) for 24 h, further evaluation was conducted to evaluate cell apoptosis and caspase-3 activity. The results in Figure 3 revealed a dose-dependent increase in cell apoptosis after carvacrol treatment. Compared with untreated cells, different concentrations of carvacrol (100, 150, 200 μM) increased apoptosis by 0.28, 0.14 and 1.15%, respectively.

Figure 4 demonstrated that caspase-3 activity in carvacrol-treated MDA-MB-231/Dox cells was also dose dependent. The activity was notably elevated by 13.90, 41.60, and 60.90% at 100, 150, and 200 μM carvacrol, correspondingly, in contrast to the control group.

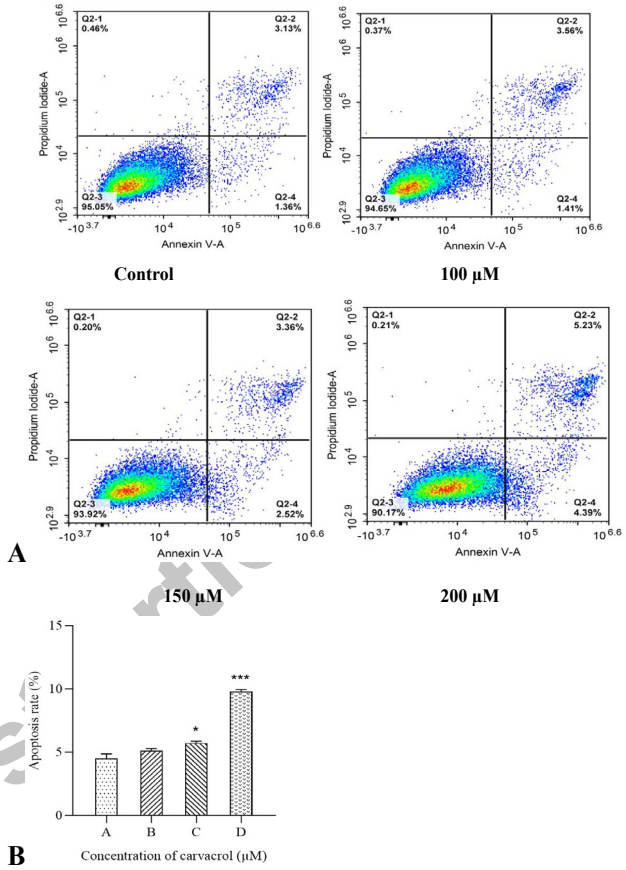


Fig. 3. Effect of carvacrol on MDA-MB-231/Dox cell apoptosis. Cells were exposed to 0, 100, 150 or 200 μM carvacrol for 24 h. Values were presented as mean ± SD, symbol asterisk indicates significant (* $p < 0.05$, *** $p < 0.001$) difference as compared to the control cells. V-viable cells; A-apoptotic cells; D-dead cells.

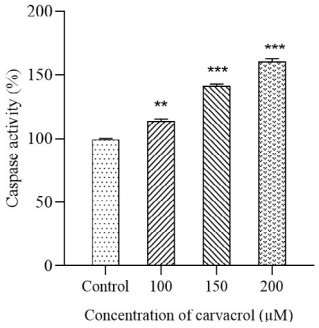


Fig. 4. Dose-dependent activation of caspase-3 in carvacrol-treated MDA-MB-231/Dox cells. Caspase-3 activity percentage in MDA-MB-231/Dox cells treated with different concentrations of carvacrol for 24 h was determined by caspase-3 activity assay. Values are presented as mean ± SD, and symbol asterisk indicates significant (** $p < 0.01$, *** $p < 0.001$) difference as compared to the control cells.

*Carvacrol regulated apoptosis-related proteins by inactivating the PI3K/Akt signaling pathway*

To further elucidate how carvacrol affects MDA-MB-231/Dox cell apoptosis, related protein expression was assessed through WB analysis. The groups treated with 100, 150 and 200 μM carvacrol exhibited decreased expression of Bcl-2, PI3K, and p-Akt, but increased Bax levels, compared to the untreated group. These differences exhibited a dose-dependent trend (Fig. 5). The findings imply that MDA-MB-231/Dox cells may drug re-sensitization upon carvacrol treatment by deactivating PI3K/Akt signaling pathway.

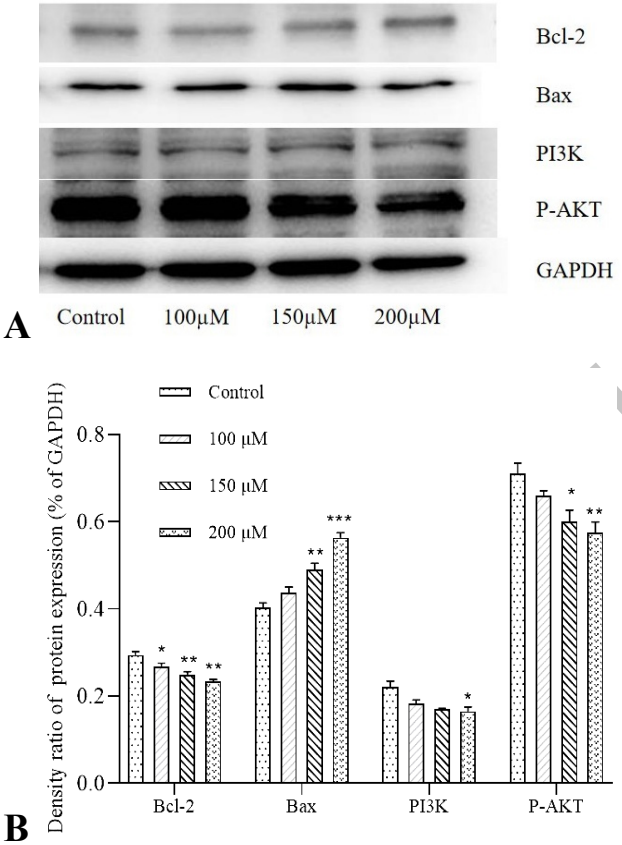


Fig. 5. Effect of carvacrol on apoptosis-related protein expressions by Western blotting. GAPDH was used as a loading control. Values are presented as mean ± SD, and symbol asterisk indicates significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) difference as compared to the control cells.

## DISCUSSION

It is well known that the emergence of Dox-resistance is the main reason for the failure of clinical Dox-resistant TNBC chemotherapy. To evaluate carvacrol's influence on Dox-resistant MDA-MB-231/Dox *in vitro*, a Dox-resistant cell model was developed in the laboratory by gradually increasing the Dox concentration, resulting in the successful establishment of a Dox-resistant cell line (MDA-MB-231/Dox), with an RI of 4.43. This study also investigated the long-term stability of this resistance. Dox IC50 values against parental and Dox-resistant cells were 2.37 ± 0.12 μM and 10.51 ± 0.23 μM, respectively, after 3 months of growth in the absence of Dox. The initial Dox IC50 was not significantly different between the parental and resistant cell lines. Multiple *in vitro* studies have employed the Dox-resistant cell model of breast cancer to explore potential therapeutics for treating Dox-resistant breast cancer (Amornsupak *et al*., 2014; Chakravarty *et al*., 2016; Chen *et al*., 2018b). Schisandrin A could reverse Dox resistance in a human breast cancer (HBC) cell line via inhibition of STAT3 and p65 phosphorylation (Zhang *et al*., 2018), while gambogic acid exhibited inhibitory properties against Dox resistant HBC cells (Wang *et al*., 2015).

Cell cycle deregulation leads to aberrant cell proliferation (Evan and Vousden, 2001; Williams and Stoeber, 2012). Dehydrozingerone treatment of human colon cancer HT-29 cells caused a dose-dependent G2/M phase arrest (Yogosawa *et al*., 2012). Carvacrol has been shown to inhibit proliferation of MDA-MB-231 cell by suppressing DNA synthesis in S-phase (Arunasree, 2010). Our study revealed that carvacrol dose-dependently inhibited proliferation of MDA-MB-231/Dox cells and induced arrest of cell cycle at G0/G1 phase, suppressed DNA replication in S phase, increased caspase-3 activity, and subsequently induce MDA-MB-231/Dox cell apoptosis. Furthermore, the apoptosis rate in MDA-MB-231/Dox cells increased with increasing concentration of carvacrol after 24 h of exposure, confirming that carvacrol can dose-dependently induce cell apoptosis.

The disruption of balance of cell proliferation and cell death is a major contributor to tumorigenesis and tumor growth (Hao *et al*., 1998). Death of cancer cells through programmed cell death or apoptosis remains a target in clinical cancer therapy (Carneiro and El-Deiry, 2020). Bcl-2 protein family, which comprises both pro- (e.g., Bax or Bak) and anti- (e.g., Bcl-2 or Bcl-xL) apoptotic members, is important in regulating apoptotic cell death, tumorigenesis and the cellular response to cancer therapies (Carneiro and El-Deiry, 2020; Qian *et al*., 2022). Increased PI3K/AKT pathway activity attenuates chemotherapy-driven apoptosis by decreasing Bax levels while increasing Bcl-2 and XIAP levels (Liu *et al*., 2020). PI3K/AKT signaling is often overactive in MDR tumors and is instrumental in promoting cellular proliferation and survival (Neophytou *et al*., 2021). Our investigation

revealed that carvacrol exposure resulted in reduced Bcl-2 levels and increased Bax levels and caspase-3 activity in MDA-MB-231/Dox cells. Hence, the possibility remains that carvacrol-induced cell apoptosis may be associated with the mitochondrial apoptotic pathway. The results reported by Arunasree *et al.* show that the anticancer properties of carvacrol are in line with our findings (Arunasree, 2010). Meanwhile, PI3K and p-Akt protein levels were diminished in the carvacrol groups, which also indicated that carvacrol treatment could induce apoptosis in MDA-MB-231/Dox cells by inactivating the PI3K/Akt signaling pathway. Further studies are necessary for a more complete understanding of how carvacrol interacts with other chemotherapy agents. Additionally, the safety and efficacy of carvacrol as an adjunct in treating breast cancer must be investigated (Crovella *et al.*, 2023).

## CONCLUSION

In conclusion, carvacrol exhibits potent antiproliferative activity, induces G0/G1 cell cycle arrest, and stimulates cell apoptosis in MDA-MB-231/Dox cells via PI3K/AKT signaling downregulation, suggesting carvacrol has the potential to be a promising candidate candidate for Dox-resistant breast cancer drug development, but its efficacy must be validated through further research. In contrast to the complex tumor microenvironment, artificially regulated *in vitro* experiments are not exactly equivalent to the internal environment of animals. Therefore, further *in vivo* research is needed.

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*Statement of conflicts of interest*

The authors have declared no conflict of interest.

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