Research Article

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A study to investigate the anticancer potential of carvacrol via targeting Notch signaling in breast cancer

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Abstract: Breast cancer (BC) continues to be a primary worldwide health concern despite the tremendous efforts made to deploy novel chemotherapeutic techniques for the treatment of BC. It is, therefore, essential to elucidate better plant-based compounds targeting deregulated signaling components in various cancer cell types. Our objective was to elucidate a potent targeted therapeutic approach by exploiting the anticancerous potential of carvacrol in MDA-MB-231 cells via employing silicon and *in vitro* approaches. *In silico* analysis was executed to identify the anticancer potential of carvacrol against BC via targeting crucial signaling component of the NOTCH pathway, namely Jagged-1 and its downstream target cyclin D1. *In vitro*, assays were also employed to display the antiproliferative potential of carvacrol at the mRNA level in MDA-MB-231 cells via targeting Jagged-1 and cyclin D1 genes. Docking studies using CB DOCK displayed better binding energy of carvacrol (Jagged-1: −5.0 and cyclin D1: −5.8) in comparison to the standard drug, 5-fluorouracil (Jagged-1: −4.5; cyclin D1: −4.6) against these crucial targets. Carvacrol potentially downregulated the expression of these crucial genes along with caspase-mediated apoptosis induction. However, more *in vitro* assays must be employed to validate its candidature for drug development against BC. This study provided a novel insight into the targeted therapeutic approach using natural products and deregulated signaling components for managing breast carcinoma.

Keywords: NOTCH signaling, breast cancer, caspase, apoptosis, CB-DOCK, carvacrol

1 Introduction

Numerous research studies have focused on targeted therapy approaches that use plant products and altered signaling components to treat breast cancer (BC) effectively [1,2]. Patients with cancer have demonstrated significant limitations with traditional therapeutic techniques, such as surgery and radiation therapy [3,4]. Therefore, it is critical to look for more non-toxic therapeutic options for the efficient treatment of BC for clinical objectives. Numerous research has deciphered the anticancer potential of carvacrol via modulating deregulated signaling components that govern angiogenesis [5], inflammation [6], apoptosis [7], and cancer cell growth [8]. Carvacrol is a monoterpene [9] that has garnered much attention lately due to its several biological benefits, including antioxidant, anti-inflammatory, neuroprotective, antitumor, and antibacterial [10]. BC progression has been linked to multiple aberrant Notch signaling components [11]. Notch components are expressed more frequently as BC advances, according to several investigations [12]. This pathway significantly affected apoptosis, cell survival, and proliferation [13]. One of the essential signaling mechanisms in drug-resistant cancer cells is the Notch pathway, as confirmed by earlier studies [14,15]. Moreover, inhibiting this pathway decreases the growth and spread of BC cells by increasing drug sensitivity [16].

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Several crucial targets of Notch signaling, including Notch receptors and respective ligands, are being explored as prognostic biomarkers in numerous human carcinomas [17–19]. These facts have further motivated us to target the ligand of Notch receptor (Jagged1) and cyclin D1 with carvacrol to elucidate a plant-based compound as a potent drug candidate for BC management. Carvacrol is used in perfumery and cosmetics, as well as for the preparation of racemic menthol. As a phenol, carvacrol possesses highly pronounced antimicrobial, antioxidant, and other biological properties [20–22]. Additionally, this research is intended to investigate the mechanism associated with the inhibitory potential of carvacrol by examining expression levels of Jagged-1, cyclin D1, and caspases in carvacrol-treated MDA-MB-231 cells. The present study would demonstrate the possible mechanism behind the anticancerous potential of carvacrol via modulating these crucial Notch signaling components in MDA-MB-231 cells. Altogether, this study would be highly beneficial for considering carvacrol as a potential therapeutic candidate for treating human BC.

2 Materials and methods

2.1 *In silico* analysis

Carvacrol was utilized as a ligand to target one of the most crucial targets of the deregulated NOTCH pathway (Jagged-1) and its direct downstream target (cyclin D1) to investigate the inhibitory potential of carvacrol in BC cells. The PDB file of these targets (Jagged1:2VJ2 and cyclin D1:3ay5) was downloaded from rcsb.org as a crystal structure (https://www.rcsb.org/). The criteria behind selecting these structures were the lowest energy. CB Dock server (https://cadd.labshare.cn/cb-dock2/index.php) was used to identify the binding efficacy of carvacrol against these target proteins by examining their binding sites and ligand stability. The complex structure was visualized using chimera software (UCSF). Additionally, the drug-likeness properties of carvacrol and standard drug (5-FU [5-fluorouracil]) were also investigated by employing the Swiss ADME tool (http://www.swissadme.ch/).

2.2 Investigation of cell viability by MTT assay

MDA-MB-231 cancer cells (NCCS, India) were cultured in DMEM growth media for 24 h at 37°C with 5% CO_2. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [23] was used to assess how well carvacrol inhibited MDA-MB-231 cells. Following a 24-h incubation period at 37°C, MDA-MB-231 cells were subjected to carvacrol treatment at concentrations ranging from 0 to 65 M. The cells were then incubated for a further 24 h. Each well was incubated for 4 h at 37°C after adding MTT dye. After that, a DMSO solution is created by gently shaking the formazan (purple-colored precipitate). Cell survival was assessed by measuring the microplate reader's final absorbance (at 490 nm).

2.3 Real-time PCR to analyze the modulation of target genes in carvacrol-treated cells

Carvacrol-treated MDA-MB-231 cells or DMSO control cells were used to measure target gene mRNA expression to determine whether carvacrol had any modulatory influence on apoptotic (anti-apoptotic or pro-apoptotic) gene transcription. TRIzol Reagent (Invitrogen manufacturer's procedure) was used to extract total RNA from carvacrol-treated MDA-MB-231 cells 24 h post-treatment. The Platinum Taq DNA polymerase kit (Invitrogen) was used to perform one-step RT-PCR using SuperScript III [24]. The relative expression of the treatment and control samples was assessed using the $2^{(-\Delta\Delta Ct)}$ technique, normalized to β-actin mRNA.

Target mRNA expression = $2^{-[(Ct \text{ of the gene of interest}) - (Ct \text{ of internal control})]}$, where Ct stands for threshold cycle for every transcript (Table 1).

2.4 Investigation of caspases (3, -8, and -9) activity in carvacrol-treated cancer cells

Colorimetric Assay Kit (BioVision) was used to measure the activity of caspases (3, -8, and -9) in MDA-MB-231 cells treated with carvacrol. Untreated MDA-MB-231 (3×10^6) or carvacrol-treated cells were incubated with a chilled cell lysis solution for 10 min (on ice). After 1 min of centrifuging lysed cells at 10,000 × *g*, the leftover supernatant was saved for additional examination. After that, 50 µl of cell lysate was combined with 10 mM dithiothreitol

Table 1: Selected primers used in this study

Gene	Forward primer	Reverse primer
Cyclin-D1	AGACCTGCGCGCCCTCGGTG	GTAGTAGGACAGGAAGTTGTTC
Jagged1	ACTGGCACGGTTGTAGCACTG	TGGTTAATGGTTATCGCTGTATCTG
β-Actin	GTCTGTGATGCCCTTAGATG	AGCTTATGACCCGCACTTAC

reaction buffer in 96-well plates. Each well was then filled with 5 µl of N-acetyl-Asp-Glu-Val-Asp-p-Nitroanilide substrate and incubated for an hour at 37°C. Using absorbance at 405 nm, the percentage change in caspase activity was determined [25].

2.5 Hoechst 33342 staining for analysis of nuclear morphology

Hoechst 33342 staining was utilized to perform a morphological evaluation of induced apoptosis in carvacrol-treated cells, as per Guo et al. [26]. To sum up, a 12-well plate was cultured with 5×10^4 cervical cancer cells (per well) overnight. The cervical cancer cells were treated with various doses of carvacrol for 6 h after reaching 70–80% confluency. This was followed by PBS washing and staining with 5 mg/ml Hoechst 33342 for 10 min at room temperature (37°C) in a humidified dark chamber. The nuclear structure of cervical cancer cells treated with carvacrol was evaluated using an inverted fluorescence microscope (ECLIPSE Ti-S).

2.6 Statistical analysis

GraphPad Prism software (version 7.0) [26] was used to analyze the experimental data. Statistical analyses were performed using one-way ANOVA. Error bars for SEM are shown. Where indicated in the figures, degrees of *p*-value significance are as follows: $*p < 0.01$ and $**p < 0.001$.

3 Results

3.1 Docking analysis of carvacrol against the crucial targeted protein of BC

The pharmacokinetic analysis of carvacrol was determined using the available online software Swiss ADME (Table 2). Lipinski's rule of five shows a compound's molecular characteristics, which are crucial for lead selectivity and optimization of a prospective orally active medication in clinical applications. An orally active chemical should not typically have more than one Lipinski violation if its bioavailability is jeopardized. Carvacrol has not demonstrated Lipinski's violation, blood–brain barrier (BBB) permeability, or permeability-glycoprotein (P-GP) substrates [27]. P-GP is an ATP-dependent bioavailability protein pump that removes medications from biological systems. The pharmacokinetics and survivability of pharmaceutical medicines are reduced by the natural release of pharmaceuticals back into the stomach lumen via PGPp (which are supposed to be PGPp substrates) [28]. CB Dock, an online docking server, was utilized for the docking investigation of carvacrol against three crucial targets of the notch signaling pathway in BC. Tables 3 and 4 show the comparative *in silico* analysis of carvacrol against three target proteins. These preliminary findings revealed that carvacrol showed a more binding affinity with the targeted

Table 2: Physicochemical properties of carvacrol (ligand)

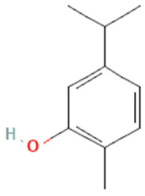
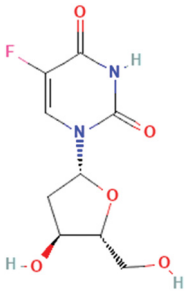
Physicochemical properties		
Parameters	**Carvacrol**	**5-FU**
Structure		
PUBCHEM ID	10364	3385
Canonical SMILE	CC1=C(C=C(C=C1)C(C)C)O	C1=C(C(=O)NC(=O)N1)F
Formula	$C_{10}H_{14}O$	$C_4H_3FN_2O_2$
Molecular weight	150.22 g/mol	130.08 g/mol
Num. rotatable bonds	1	0
Num. heavy atoms	11	9
Num. H-bond donors	1	2
Num. arom. heavy atoms	6	6
Num. H-bond acceptors	1	3
Molar refractivity	48.01	27.64
Pharmacokinetics of carvacrol and 5-FU		
Log Kp	−4.74 cm/s	−7.73 cm/s
P-gp substrate	No	No
GI absorption	High	High
BBB permeant	Yes	No
Cytochrome P450 inhibitors		
CYP2C19 inhibitor	No	No
CYP1A2 inhibitor	Yes	No
CYP2D6 inhibitor	No	No
CYP2C9 inhibitor	No	No
CYP3A4 inhibitor	No	No

Table 3: Docking results of carvacrol with Notch-1 and Jagged-1 using CB Dock

Target	Carvacrol		5-FU	
	Vina score	Contact residues	Vina score	Contact residues
Jagged 1	−5.0	Chain A: ASP205 PHE206 PHE207 GLY208 HIS209 TRP224 MET225 ASN230 ARG231 ALA232 ILE233 CYS234 ARG235 GLN236 GLY237 CYS238 SER239 PRO240 GLY243 SER244 CYS245 LYS246 LEU247	−4.5	Chain A: THR286 ASN287 TRP288 ASP296 ASN298 GLY301 THR302 Chain B: ASP189 TYR190 TYR192 CYS200 ARG201 PRO202 CYS212
Cyclin D1	−5.8	Chain A: GLN193 ALA194 VAL195 GLU196 GLU197 SER198 ASP199 PRO200 LEU231 TYR232 TRP233 SER234 GLU235 GLN238 TYR296 PRO297	−4.6	Chain A: ALA194 VAL195 GLU197 SER198 ASP199 PRO200 GLN229 ASP230 LEU231 TYR232 TRP233 SER234 GLU235 ASP237 GLN238 PRO297 PRO298 MET299

protein than the standard drug 5-FU. However, further studies are still needed to validate these binding affinities.

3.2 Carvacrol reduced cell viability in MDA-MB-231 BC cells

The MTT assay assessed the efficacy of carvacrol in suppressing the growth of MDA-MB-231 cells. MDA-MB-231 cancer cells were exposed to varying doses of carvacrol (0–65 μM) for 24 h. Carvacrol-treated MDA-MB-231 cells demonstrated a dose-dependent and substantial reduction in MDA-MB-231 cell viability compared to control cells (untreated) (Figure 1a). The suppressive capacity of carvacrol on MDA-MB-231 cells suggests that carvacrol may have a combined effect with 5-FU on the survival of MDA-MB-231 cells. Cell viability was potentially reduced by the combination treatment of carvacrol and 5-FU (Figure 1b). The Origin software (Data study and Graphing Software) was utilized to obtain the IC_{50} value (IC_{50} = 44.42 μM), which was used to choose the appropriate doses for further analysis.

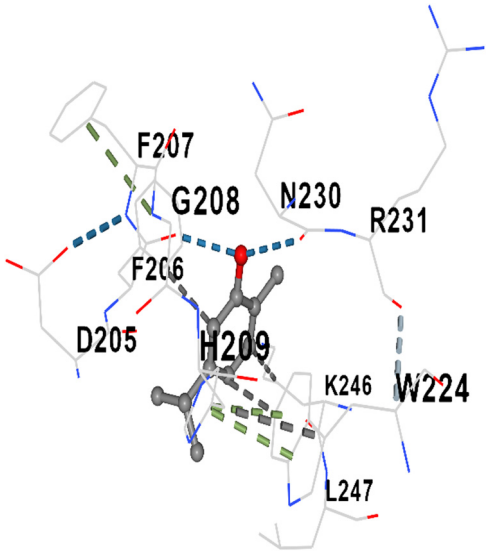
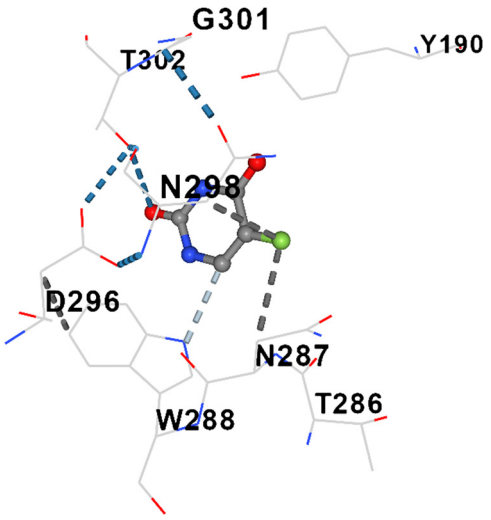
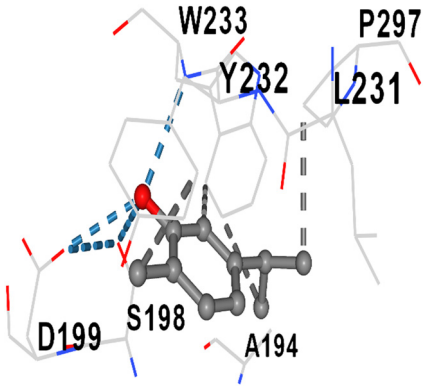
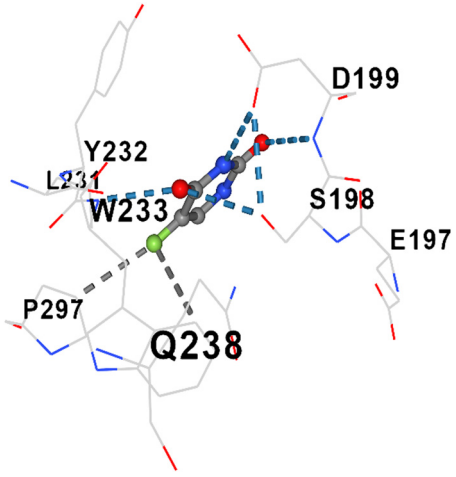
3.3 Effect of carvacrol on modulation of Jagged-1 and cyclin D1 mRNA expression in MDA-MB-231 cells

To elucidate the mechanism underlying apoptosis in carvacrol-treated MDA-MB-231 cancer cells, we employed RT-PCR to examine the mRNA transcript level of apoptosis-regulating genes. Carvacrol may modify the amounts of Jagged-1 and cyclin D1 mRNA transcripts, which are oncogenes associated with BC and influence the course of the cell cycle. The data demonstrated that carvacrol-treated MDA-MB-231 cells had substantially lower cyclin D1 and Jagged-1 expression (mRNA level) (Figure 2). Overall, these results supported the earlier research showing that carvacrol therapy decreased the expression of cyclin D1 (Jagged-1's immediate downstream target) and Jagged-1 in MDA-MB-231 cancer cells.

3.4 Carvacrol-induced caspase (9, -3, and -8) activation and apoptosis induction in MDA-MB-231 cancer cells

Caspases are a group of cysteine proteases that initiate apoptosis by cleaving proteins at specific aspartic acid residues. Hence, we examined whether the induction of apoptosis in MDA-MB-231 cancer cells by carvacrol resulted

Table 4: Docked images of carvacrol and 5-FU with Notch-1 and Jagged-1

	Docked complex	
Target	**Carvacrol**	**5-FU**
Jagged-1		
Cyclin D1		

from nuclear morphology alteration and caspase activation. After 24 hours, carvacrol-treated MDA-MB-231 cells showed alteration in nuclear morphology as evidenced by nuclear condensation (Figure 3a). Compared to untreated (control) MDA-MB-231 cells, Figure 3b showed a significant increase in caspase-3, -8, and -9 activity. Therefore, it was observed that MDA-MB-231 cells treated with carvacrol showed nuclear condensation and dose-dependent increase in caspase activity. Taken as a whole, our findings offer strong evidence for the crucial role that caspase activation plays in carvacrol-induced apoptosis.

4 Discussion

Jagged canonical Notch ligand 1 (Jagged-1) is highly expressed in various cancer types and plays a significant role in the Notch signaling system, which is closely associated with tumor biology [29,30]. The correlation between the levels of Jagged-1 mRNA and the overall survival of cancer patients has garnered increasing interest in understanding the role of Jagged-1 in BC. When compared to localized non-metastatic BC, Jagged-1 expression was significantly higher in metastatic BC tissues (bone, liver, lung, and brain metastases), and a high

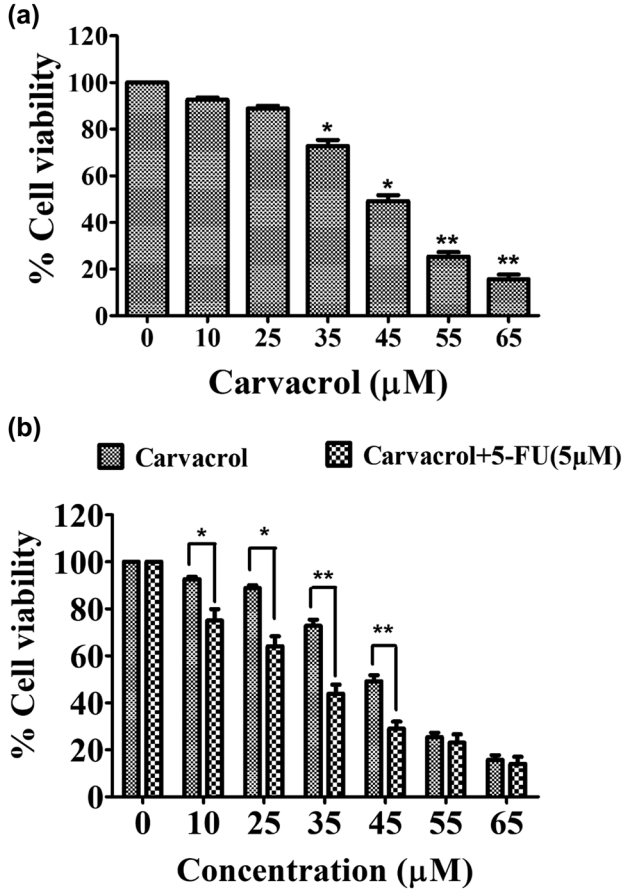


Figure 1: MTT assay in carvacrol-treated MDA-MB-231 cells after 24 h of treatment (a) carvacrol reduced the cancer cell viability (b) combined effect of carvacrol + 5-FU. SEM and means from three separate trials were shown; *p < 0.01 and **p < 0.001 were given in contrast to corresponding control values.

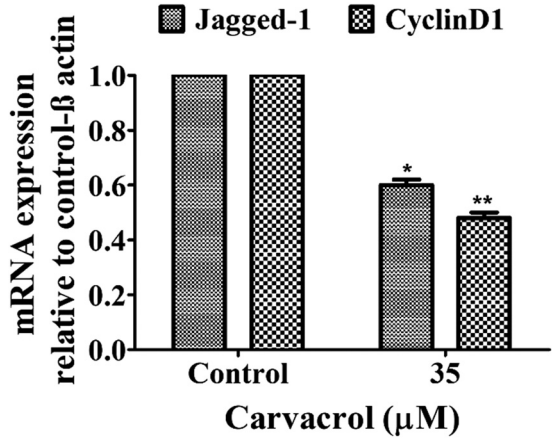


Figure 2: Modulation of target gene and protein expression levels in carvacrol-treated MDA-MB-231 cells. mRNA expression of target genes in treated cells was analyzed using RT-PCR. Three independent experiments' SEM and mean values were provided: *p < 0.01, **p < 0.001 in contrast to the corresponding control value.

positive rate of Jagged-1 was correlated with malignant and invasive features of tumors, suggesting that Jagged-1 plays a significant role in BC metastasis [31]. Carvacrol has been shown to have anti-inflammatory, antioxidant, and anticancer properties in several cancer models [32]. We have been motivated to examine the inhibitory capacity of carvacrol against Jagged-1 in MDA-MB-231 cancer cells using *in silico* and *in vitro* experiments, which have not been previously investigated.

Our investigation found that carvacrol exhibited a substantial decrease in binding energy against Jagged-1, a protein known to be associated with the advancement of BC. Cyclin D1 is a nuclear protein present in the nucleus during the G1 phase of the cell cycle and is absent from the nucleus during the S phase [33]. Cyclin D1 is a prominent cell cycle regulator strongly linked to being a proto-oncogene in several human tumors, such as breast carcinomas [34]. Cyclin D1 has been reported as one of the direct downstream targets of Jagged-1 [35]. Therefore, we conducted an *in silico* investigation to determine the effectiveness of carvacrol against cyclin D1. The investigation of carvacrol's docking with cyclin D1 has revealed a substantial binding energy, indicated by a negative value.

The precise molecular mechanism by which carvacrol induces cell death in BC by targeting key dysregulated components of the notch pathway remains unclear. The *in silico* findings were confirmed using *in vitro* experiments involving MTT, RT-PCR, caspases, and Hoechst analysis. The MTT experiment revealed that carvacrol exhibited substantial inhibitory effects against the growth of MDA-MB-231 cancer cells at a highly potent dose. In addition, we conducted RT-PCR research to confirm the substantial binding affinity of carvacrol toward these essential targets. These results indicate that carvacrol has significantly reduced the expression levels of Jagged-1 and cyclin D1 in BC cells.

We also emphasized examining the potential of carvacrol to induce apoptosis and its correlation with apoptotic pathways in MDA-MB-231 cells treated with carvacrol. The condensed or fragmented nucleus is a crucial indicator of apoptotic induction [36]. The data we obtained coincide with the previous fact, which showed that carvacrol-treated MDA-MB-231 cells exhibited fragmented or condensed nuclei, as confirmed by Hoechst's analysis. This finding suggests carvacrol-induced cell death by apoptosis. Carvacrol treatment in MDA-MB-231 cancer cells activated markers indicating programmed cell death and the emergence of toxic effects. The activation of caspases is a critical mechanism involved in initiating apoptosis, which can be controlled by cytochrome c [37]. Our investigation found that carvacrol treatment increased the expression of caspase-3, -9, and -8. These results

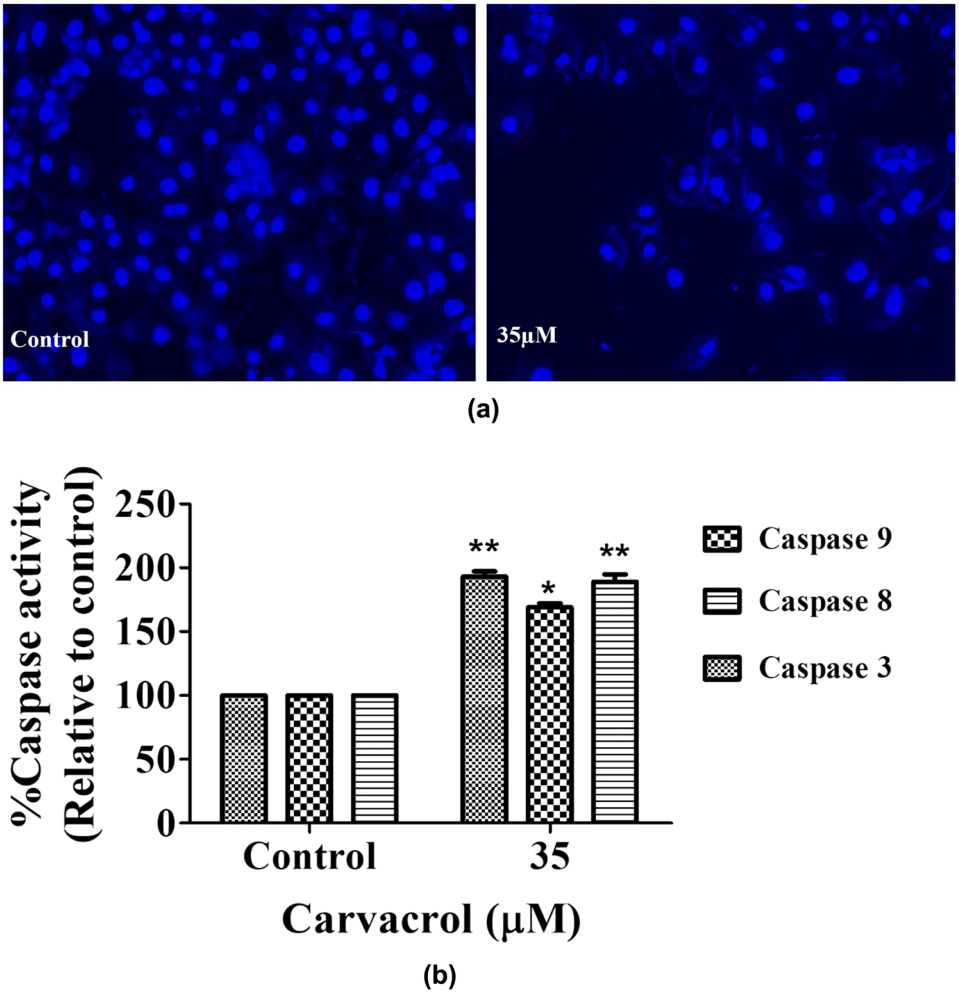


Figure 3: Effects of carvacrol administration on (a) apoptosis using HOECHST analysis and (b) caspases (9, -3, and -8) cleavage in MDA-MB-231 cancer cells treated for 24 h. SEM and mean values from three separate trials were shown; *$p < 0.01$, **$p < 0.001$ were given in contrast to the corresponding control values.

indicate that carvacrol has the potential to trigger apoptosis by either the mitochondria-mediated pathway or the extrinsic pathway. It has not yet been demonstrated that carvacrol and Jagged-1 expression in MDA-MB-231 cancer cells are inversely correlated. Thus, apoptotic induction in carvacrol-treated MDA-MB-231 cells may be caused by these modulatory effects (down-regulation of Jagged-1 and cyclin D1). Altogether, these studies have provided compelling evidence that carvacrol, which targets the Jagged-1 and cyclin D1 genes and proteins, has excellent potential as a curative candidate for cervical cancer.

5 Conclusion

Various factors, such as genetics and diet, affect the incidence of BC. Multiple molecules and diverse signaling pathways, such as apoptosis, oxidative stress, and inflammation, are involved in the development and advancement of this condition. Several studies have shown evidence for the possible role of carvacrol in a range of human cancer conditions. This polyphenol molecule has several properties, including anti-inflammatory, anti-angiogenesis, apoptotic inducer, and antioxidant properties. The dysregulation of Notch signaling has played a critical role in BC progression by facilitating proliferative signaling, evading tumor suppressors, activating telomerase, inducing angiogenesis, and promoting metastasis. In conclusion, our research suggests that carvacrol significantly inhibited Jagged-1 and cyclin D1 expression, induced caspases (-3, -8, and -9) activation and apoptosis induction in MDA-MB-231 cancer cells. Hence, this research would contribute to our understanding of the molecular mechanisms by which carvacrol inhibits the growth of human BC cells, which would further motivate

future cancer researchers to develop a potential drug candidate for this deadly malignancy. However, additional investigations are still required to verify carvacrol as a strong contender for effectively managing BC.

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Conflict of interest: The authors declare no conflict of interest, financial or otherwise.

Ethical approval: The conducted research is not related to either human or animal use.

Data availability statement: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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