ORIGINAL RESEARCH

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Carvacrol is a Novel Natural Therapeutic Approach Through the Inhibition of Proliferation, Autophagy and Migration in Pancreatic Ductal Adenocarcinoma Cells

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Abstract

Objective

Pancreatic ductal adenocarcinoma, which is the most common and aggressive pancreatic cancer, has the highest mortality rate of cancers because of difficulties in diagnosis and chemoresistance. As the chemotherapeutic options are very limited and insufficient for PDAC, novel effective therapeutic approaches are urgently needed for pancreatic cancer patients. Carvacrol naturally found in thyme (*Thymus vulgaris*), wild bergamot (*Citrus aurantium var. bergamia Loisel*), black cumin (*Nigella sativa*), marjoram (*Origanum scabrum, Origanum microphyllum, Origanum onites, Origanum vulgare*) and black pepper (*Lepidium flavum*) plants is shown to have antibacterial and antioxidant effects. In this study, we aimed to investigate the anticarcinogenic potential of carvacrol through proliferation and autophagy in PDAC cells.

Material and Method

To determine the anti-proliferative effects of carvacrol in Panc-1 cells, we performed the MTS assay using different carvacrol doses of 100, 200, 300, 400, 500, 600, 700 and 800 µM at 24h, 48h, 72h. The gene and protein expressions of Atg16L1 and Beclin-1, autophagy key mediators, were analyzed by RT-PCR and western blot in Panc-1 cells treated with 300 and 400 µM for 24h, 48h. Additionally, the migrative property of PDAC cells was evaluated using a wound healing assay.

Results

Based on MTS results, carvacrol significantly inhibited cell proliferation in the doses of 300 and 400 µM at 24h and 48 h in Panc-1. These same doses led to decreased autophagy and migration through, Atg16L1, and Beclin-1 expressions.

Conclusion

Our findings first revealed that carvacrol has promising value as a potential therapeutic approach for PDAC. We believe that further mechanistic investigations will be a guide for its clinical usage.

Keywords: Pancreatic cancer, carvacrol, proliferation, autophagy, migration

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Introduction

The pancreas, though varying in mass and volume from person to person, is divided into five anatomical sections: the head, uncinate process, neck, body, and tail, separated by fine connective tissue. It has two main functions exocrine and endocrine. In its endocrine function, it synthesizes hormones such as glucagon, insulin, and somatostatin, while in its exocrine function, it produces digestive enzymes like lipase, amylase, and trypsin. The homeostatic balance is maintained by the regeneration of intact pancreatic tissue. However, this balance is disrupted by factors such as inflammation and tissue damage, causing the acinar cells responsible for the exocrine function to undergo morphological and genetic differentiation into ductal structures through the Acinar-Ductal Metaplasia (ADM) process, leading to pancreatic intraepithelial neoplasia (PanIN) and eventually tumor formation. Pancreatic tumors include those originating from the endocrine or exocrine components of the pancreas, with pancreatic adenocarcinoma being the most common and aggressive type originating from the exocrine pancreas. Among these, pancreatic ductal adenocarcinoma (PDAC) is the most frequently seen cancer type, 90%, in the pancreas, especially when compared to acinar carcinomas and neuroendocrine tumors (1–3).

When examining the molecular mechanisms and mutations of PDAC cells, several key mutations and signaling pathways are found to play significant roles in the development and progression of this cancer type. A point mutation that activates the KRAS oncogene, a member of the RAS family and a GTPase, leads to its persistent binding to GTP, triggering the onset of cancer via three signaling pathways: the RAF/ERK pathway, phosphoinositide 3-kinase (PI3K), and RalGDS. This mutation is observed in low-grade PanINs. Mutations in the RAF family, activated by RAS through progressive phosphorylation, lead to the activation of the mitogen-activated protein kinase ERK (MAPK-ERK), contributing to the uncontrolled proliferation of cancerous cells. Thus, in the medullary type of PDAC, RAF mutations replace KRAS mutations as the primary cause. Mutations in tumor suppressor genes such as TP53, CDKN2A, and SMAD4 further the disease progression. The Transforming Growth Factor Beta (TGF-β) signaling pathway manages basic cellular processes. Binding of the tumor-suppressive SMAD4, encoded by the DPC4 gene, to TGF-β receptors on the cell surface activates SMAD proteins, which form a complex with SMAD4 to regulate gene activity in specific DNA regions, effectively triggering cell cycle and apoptosis. However, mutations in these proteins inactivate them, playing a role in cancer progression (4,5). Due to high metastatic potential, the enhanced autophagy results in PDAC cells (6,7). It is well known that autophagy serves as a survival mechanism in many cancer cells, including pancreatic cancer, in contrast to cell death (8,9). Unless autophagy is inhibited in cancer cells, apoptosis cannot be activated. Therefore, key autophagic mediators such as Beclin-1, ATG proteins, and LC3-II are overexpressed in PDAC cells (7,10). All these mutations and dysregulations in these mediators lead to uncontrolled cell division, playing a critical role in cancer pathogenesis and contributing to changes in the microenvironment of cancer cells, increasing their resistance to chemotherapy and proliferation (11).

One of the factors making PDAC so aggressive and resistant to chemotherapy is thought to be the desmoplastic tissue structure, consisting of activated fibroblasts, nerve tissue, and collagen fibers. Desmoplasia not only creates oxidative stress in cells but also reduces nutrient and drug availability. Consequently, PDAC cells reprogram their metabolism to adapt to these processes, increasing their resistance to conventional chemotherapy (12).
Current treatment for pancreatic cancer primarily involves surgical removal of the tumor. If surgery is not possible due to the tumor's conditions, treatment is attempted through three methods, which are radiation therapy combined with chemotherapy, or chemotherapy alone in the case of metastasis (13). Despite these treatment methods, the overall survival rate remains very low due to the resistance of cancer cells to chemotherapy drugs, highlighting the urgent need for developing alternative treatment methods. Research into the antiproliferative activity of natural products is crucial in this regard (14).

Carvacrol (CV; C10H14O; also known as 5-iso-propyl-2-methylphenol by the International Union of Pure and Applied Chemistry) is a liquid phenolic monoterpenoid and primarily found in the essential oil of oregano (*Origanum vulgare*). It is commercially synthesized by chemical and biotechnological techniques and is highly soluble in acetone, ether, and diethyl ether due to its lipophilic nature (14,15). Carvacrol is known for its antibacterial properties against almost all Gram-negative and Gram-positive bacteria, except for the hospital pathogen *Pseudomonas aeruginosa*. It inhibits the toxin synthesis and growth of foodborne pathogens such as *Bacillus cereus, Escherichia coli*, and Salmonella and also prevents biofilm formation in fungi like Candida, adding to its antifungal properties. Apart from these benefits, the investigations in vitro

and in vivo have revealed its anticancer properties through the apoptosis mechanism (16,17).

Despite these studies, there is no mechanistic-level research on the anti-carcinogenic efficacy of carvacrol in pancreatic cancer. Therefore, this study aims to investigate the effects of carvacrol on autophagy and cell proliferation, key mechanisms in the progression of PDAC cells, contributing to the literature and science. While literature reviews have revealed studies on carvacrol's effects on apoptosis and oxidative stress in various cancers, there is almost no information on its autophagy mechanism in pancreatic cancer, making this research pioneering in the field.

Material and Method

Cell Culture

Human PDAC cell lines having high metastatic potential (Panc-1) and non-tumorigenic human pancreatic epithelial cell line (HPDE) were cultured using Dulbecco's Modified Eagle Medium (DMEM) with different glucose concentrations specific to each cell line. Panc-1 cells were cultured in high glucose DMEM media (Invitrogen, Carlsbad, CA, USA), while HPDE in special media containing 75% of low glucose DMEM (Cegrogen Biotech, Stadtallendorf, GERMANY) supplemented with 10% FBS and 25% of M3:BaseFTM supplemented with growth factors (Incell; San Antonio, TX, USA), in the presence of 1% Penicillin/Streptomycin (Invitrogen; Carlsbad, CA, USA) under regular culture conditions, at 37 °C in a water-saturated 95% air and 5% CO2 atmosphere. When the cell density on the flask surface reached 80%, the cells were washed with 1X Dulbecco's PBS (Phosphate Buffered Saline) (Cegrogen Biotech; Stadtallendorf, GERMANY) and detached using 1X 0.25% Trypsin-EDTA (Thermo Scientific; Waltham, MA, USA). The passage number of cells was used up to 15. Cell counting was performed using Trypan Blue and a Thoma Hemocytometer before setting up experiments.

Cell Viability/Cytotoxicity Assay

The principle of the cell viability/cytotoxicity assay is based on the activity of oxidoreductase enzymes, which are indicators of cell viability and mitochondrial enzymes. These enzymes depending on NAD(P)H can convert tetrazolium to colored formazan, making the viability measurable as absorbance in a spectrophotometer by the amount of formazan produced. Panc-1 and HPDE cells were distributed into 96-well sterile microplates as 4000 cells per well and incubated overnight to allow cell attachment. The viability of Panc-1 and HPDE cells was determined using MTS Cell Proliferation Assay Kit (Promega Corporation; Madison, Wisconsin, USA) in the cells treated with carvacrol in doses of 100, 200, 300, 400, 500, 600, 700 and 800 µM for 24 h, 48 h, 72 h. At the end of the incubation period, the colored formazan products were measured at the absorbance of 490 nm using a Multiskan GO spectrophotometer (Thermo Scientific).

RT-PCR (Reverse-Transcriptase Polymerase Chain Reaction) Assay

Based on the results of the cell viability assay, Panc-1 cells were seeded into 6-well sterile microplates at 500,000 cells per well and treated with carvacrol in doses of 300 and 400 µM for 24h. At the end of the treatment, cDNA was synthesized from RNA using a cDNA synthesis kit (Applied Biosystem; Vilnius, LTU) followed by total RNA isolation with TRIzol reagent (Invitrogen; Life Technologies, Carlsbad, CA). The forward and reverse primer sequences were as follows, respectively; Beclin-1 5' GAA CCG CAA GAT AGT GGC AGA 3' and 5' CAG AGC ATG GAG CAG CAA CA 3'; ATG16L1 5' TCA GAT CTT CAT TCA GTG TTG GC 3' and 5' GCT CCT GGT TCT CTT CCG TAG T 3'; and reference gene GAPDH: 5' CAA GGT CAT CCA TGA CAA CTT TG 3' and 5' GTC CAC CAC CCT GTT GCT GTA G 3'. All RNA samples were amplified in PCR program included initial denaturation at 94℃ for 2 minutes, and then 94℃ for 30 seconds, 55℃ for 45 seconds, 72℃ for 1 minute as 35 cycles, finally additional elongation at 72℃ for 5 minutes. The PCR products were loaded onto 1.2% agarose gels in 1X TBE buffer in the presence of 10X Blue JuiceTM Gel Loading Buffer (Invitrogen, Carlsbad, CA, USA) and densitometrically analyzed with Image J software (NIH). Results were expressed as fold change compared to non-treated conditions.

Wound Healing/Migration Assay

To analyze the effect of carvacrol on cell migration, a wound healing/migration assay was conducted based on the ability of cells to migrate into a created gap. After Panc-1 cells were distributed into 6-well sterile microplates as 500,000 cells per well were incubated overnight to allow cell attachment, a line with a sterile 1 mL pipette tip was marked at the bottom of each well. The cells were then treated with 300 µM and 400 µM doses of carvacrol to be able to evaluate the possible changes in cell motility and migration. Then cells were photographed using a phase-contrast microscope (Zeiss, Jena, Germany) just before (0 h) and 24 h, 48 h later of treatment. The migrated cells to the line were counted, and photographs were taken under the microscope at 0 h and 24 h, 48 h later, with at least 5 random non-overlapping areas for each.

Statistical Analyses

All data were expressed as the means ± standard error of the mean (SEM) of three independent experiments. Statistical significance was determined using the Student t-test. P values less than 0.05 were considered statistically significant. Student's t-tests and ANOVA were calculated using GraphPad software.

Results

Carvacrol Decreases the Cell Viability in PDAC cells, not in HPDE

The effects of carvacrol on the viability of PDAC cells were determined by MTS analysis in the Panc-1 cell

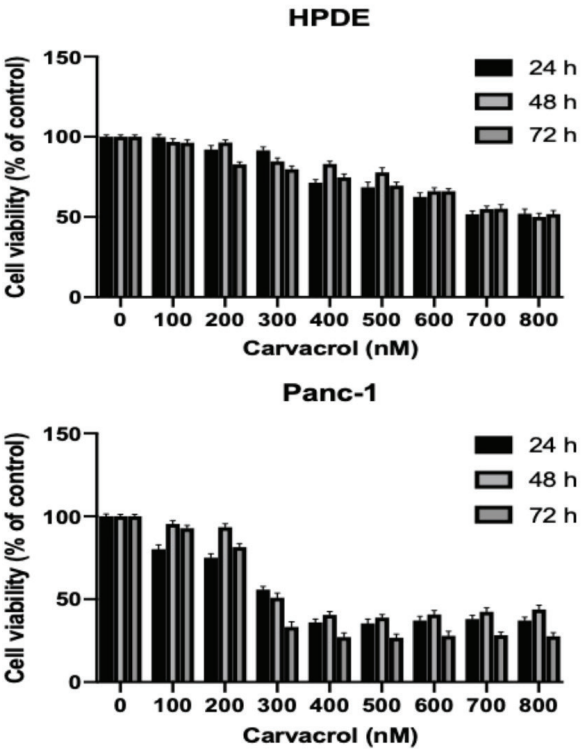

Figure 1
Carvacrol suppresses proliferation in PDAC cells, Panc-1 (A), compared to non-tumorigenic human pancreatic epithelial cells, HPDE (B). Cell proliferation was examined using the MTS assay, and the mean absorbance at 490 nm wavelength was determined at 24 h, 48 h and 72 h. Data were given as means ± SEM of three independent experiments. *p < 0.05 means significantly statistical different compared to non-treated (NT) conditions.

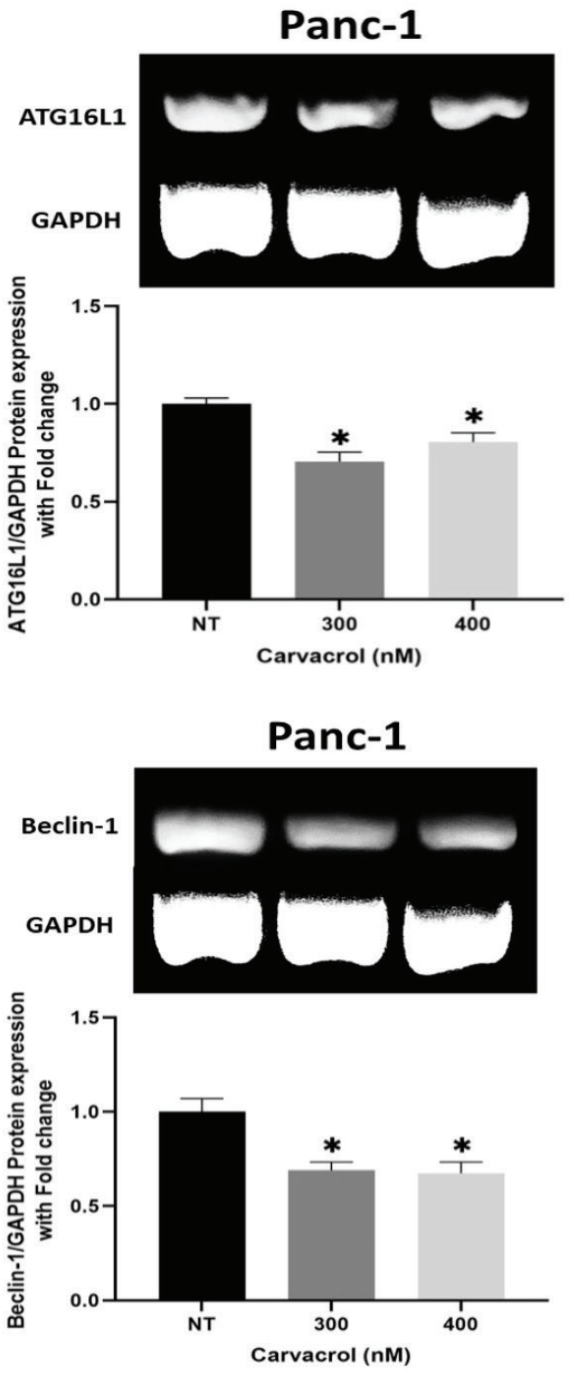

Figure 2
Carvacrol inhibits the key mediators of autophagy through ATG16L1 (A) and Beclin-1 (B) gene expressions in Panc-1 cells transfected with 300 and 400 µM carvacrol for 24h. GAPDH was used as a loading control for RT-PCR analyses. Data were normalized to the expression of loading controls and represented means ± SEM of three independent experiments. ATG16L1 and Beclin-1 gene expressions in Panc-1 were evaluated by comparing to NT conditions (*p < 0.05).

line. Of the carvacrol doses from 100 through 800 µM, the most significant carvacrol (CV) doses were found to be 300 nM and 400 µM both for 24 h and 48 h in terms of IC50 values. Panc-1 cell viability related to the treatment of 300 and 400 µM doses of carvacrol decreased to 55.8% and 36%, 50.88% and 40.5% within 24 h and 48 h, respectively, when compared to non-treated conditions, whereas 100 and 200 µM carvacrol did not cause any inhibition on cell viability, either 24 h or 48 h. In contrast to Panc-1, human non-tumorigenic pancreatic epithelial cell HPDE was not affected by carvacrol treatment. In HPDE control cells, carvacrol started to affect about 700 and 800 µM doses (Figure 1A, 1B). Therefore, 300 and 400 µM doses of carvacrol were selected for our further experiments; any adverse effects related to carvacrol occurred in HPDE.

Carvacrol Inhibits the Expression of Autophagy Mediators in Panc-1 Cells

To evaluate the anti-carcinogenic effects of carvacrol on the key oncogenic mediators, Beclin-1 and ATG-16L1, as autophagic regulators, gene expressions were analyzed by RT-PCR in the Panc-1 cell line treated with 300 and 400 µM carvacrol for 24 h. Both doses of carvacrol suppressed ATG16L1 and Beclin-1 mRNA expressions. These decreases were significantly observed, approximately 30-35% compared to non-treated conditions (Figure 2A, 2B).

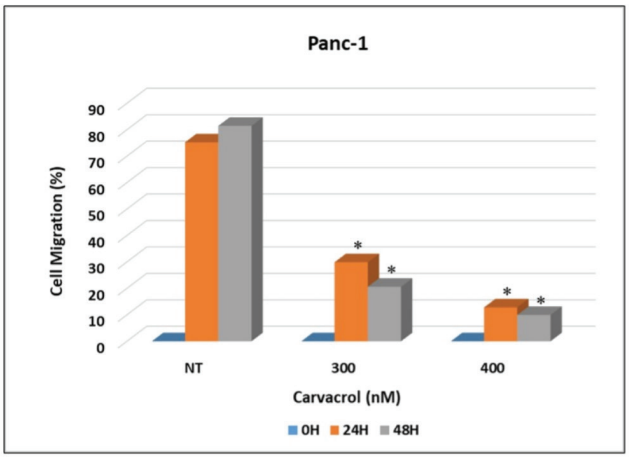

Figure 3
Carvacrol decreases migration in Panc-1 cells. Cells were counted in 5 random fields per well at 40x at 0 h, 24 h, and 48 h for migration. The migrating cells percentages of open area in the presence of 300 and 400 µM carvacrol were calculated and compared with the related controls in Panc-1 cells (*p < 0.05). Data represent means ± SEM of three independent experiments.

Carvacrol Suppresses the Migration Ability of Panc-1 Cells

Autophagy is closely associated with the aggressiveness and metastatic potential of PDAC cells. Therefore, the effect of carvacrol on cell migration was determined by the scratch wound-healing assay in Panc-1 cells. As shown in Figure 3, the distance between the edge of the wound was significantly larger than that of the control group after carvacrol treatments in a dose and time-dependent manner. Among them, a 300 µM dose of carvacrol at 24 h was observed the most effective dose because of decreased migration by 30% when compared to non-treated condition (Figure 3A, 3B).

Discussion

To the best of our knowledge, we are the first to investigate the effect of carvacrol on autophagy mediators in pancreatic cancer. In this study, we observed a 30-35% inhibition in the levels of autophagy mediators ATG-16L1 and Beclin-1 after 24 h treatments with 300 and 400 µM carvacrol in Panc-1 pancreatic ductal adenocarcinoma cells, compared to untreated condition. Additionally, we also noted nearly a 30% inhibition in the migration capability of cancer cells with a 24 h treatment of 300 µM carvacrol.

Previous studies on cancer have shown that Carvacrol-Zinc Oxide Quantum Dots (CVC-ZnO QD) suppress the expression of the anti-apoptotic protein Bcl-2 while increasing the expression of pro-apoptotic proteins Bax, caspase-9, and caspase-3 in the breast cancer cell line MDA-MB-231 (18). Additionally, high doses of carvacrol were reported to significantly inhibit cell proliferation in SW480 cells. In the hypoxic colorectal cancer cell line SW480, hypoxia was first induced by CoCl2, and then the cells were treated with carvacrol at concentrations of 400, 200, 100, 50, 25, 12.5, and 6.25 µg/mL. After 48 h treatment, the IC50 value was found to be approximately 324 µg/mL using the MTT assay. It was observed that carvacrol at concentrations of 50, 25, 12.5, and 6.25 µg/mL had a dose-dependent inhibitory effect on both cell proliferation and migration in this cell line (19).

Al-Fatlawi et al. investigated the effects of carvacrol on breast cancer cells (MCF-7), becoming the first researchers to confirm that carvacrol induces cytotoxicity and apoptosis by increasing the expression of pro-apoptotic genes. In human breast cancer cells (MCF-7), a 48 h treatment with carvacrol resulted in an IC50 value of 244.7 ± 0.71 µM. Additionally, it was reported that carvacrol induced apoptosis through the activation of the p53-dependent Bcl-2/

Bax pathway, as well as causing the expression of caspase genes (caspase-3, -9, and -6) and genomic DNA fragmentation (20).

The inhibitory effect of carvacrol on cell viability in prostate cancer cells, PC-3 and DU145, was analyzed using the CCK-8 assay. The IC50 values were found to be 498.3 µM and 430.6 µM, respectively. Carvacrol also reported was reported to reduce colony formation at doses of 250 and 500 µM in PC-3 and DU145 cell lines, respectively. Based on the related results, carvacrol has been shown to have anti-proliferative and anti-metastatic effects by blocking TRPM7 cation channel activity and acting through PI3K/Akt and MAPK/ERK signaling pathways, which led to decreased F-actin dynamics and MMP-2 protein expression (21). Compared to carvacrol, carvacrol-derived Schiff base complexes, which are carvacrol aldehyde, the Schiff base, and the copper–Schiff base complex, were found to decrease the viability of A549 cells in a dose-dependent manner as IC50 doses of 278.3 ± 4.33, 492.79 ± 4.05, and 233.49 ± 4.18 µg/mL, respectively. In addition to the inhibitory effect of cell viability, they were also found to arrest the cell cycle at the G2/M phase, reduce migration, and induce apoptosis by decreasing the expression of Bcl-2, while increasing the expression of Bax, caspase-3, and caspase-9 in A549 cell line (22).

It was observed that the proportion of cells in the S phase of the cell cycle decreased from 28.13% to 17.46% compared to untreated conditions in Tca-8113 cells, a cell line derived from clinical stage III human tongue squamous cell carcinoma, treated with 40 µM carvacrol. This effect was attributed to the inhibition of key cell cycle mediators Cyclin D1 and CDK4 and the induction of p21. Additionally, carvacrol significantly reduced colony formation at 40 µM and 80 µM concentrations compared to the control group. In the same study, the apoptotic effects of carvacrol in Tca-8113 cells were also investigated. It was found that after 24 h carvacrol treatment, the expression of pro-apoptotic proteins Bax and Cox2 decreased, and apoptotic effects were observed starting from a concentration of 20 µM, based on cell morphology. In Tca-8113, carvacrol was also found to inhibit focal adhesion kinase (FAK) activity and its signaling pathways, significantly reducing migration and invasion by decreasing the expression of ZEB1 and β-catenin proteins, as well as dose-dependently reducing MMP-2/9 protein expression (23).

In human hepatocellular carcinoma cell line HepG2, it was determined that carvacrol inhibits cell proliferation, with an IC50 value of 0.4 mmol/L. Carvacrol revealed its anti-proliferative and apoptotic effects via reducing Bcl-2 protein expression in a dose-dependent manner (24).

Apart from all these cancer types, there are very limited reports related to the potential therapeutic effects of carvacrol in pancreatic cancer. Carvacrol was first shown to have protective effects on the pancreas in acute pancreatitis by inducing endogenous antioxidant defense mechanisms and reducing malondialdehyde (MDA) levels, an indicator of oxidative stress (25). Following acute pancreatitis, Güneş et al. revealed that carvacrol exhibits anti-carcinogenic effects by reducing cell proliferation and inducing apoptosis in the Panc-1 pancreatic cancer cell line. Based on the XTT assay, the IC50 dose of carvacrol was obtained as 664.02 µM at 24 h treatment compared to the non-treated condition. This resulted from a significant increase in pro-apoptotic gene expressions such as Bax, caspase-3, caspase-8, CYCS, FADD, FAS, p53, and a decrease in Bcl-2. They also obtained the anti-metastatic potential of carvacrol because it significantly resulted in upregulating E-Cadherin, TIMP2, and TIMP3 and downregulating N-Cadherin and ZEB2 gene expressions (26).

All these reports related to the anti-carcinogenic effects of carvacrol are closely linked to our findings. Carvacrol strongly has the potential linked to anti-proliferation, anti-migration, and pro-apoptotic effects in several cancer cell lines, including PDAC, which is the most common and aggressive pancreatic cancer type. Because of the late diagnosis and frequent resistance to conventional chemotherapies of pancreatic cancer cells, there is an urgent need to provide novel natural therapeutic agents. At this point, carvacrol has promising value as a potential therapeutic approach for PDAC. We believe that further mechanistic investigations will be a guide for its clinical usage.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

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Availability of Data and Materials
Data sharing is not applicable.

Authors Contributions
ZA: Formal analysis, Writing-original draft.

NG: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing-review & editing.

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