Arabian Journal of Chemistry (2021) **14**, 103187

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Inhibition effects of 7-phloro-eckol from *Ecklonia cava* on metastasis and angiogenesis induced by hypoxia through regulation of AKT/mTOR and ERK signaling pathways

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Received 14 March 2021; accepted 25 April 2021
Available online 30 April 2021

KEYWORDS

7-phloro-eckol;
Ecklonia cava;
Hepatoma;
Hypoxia;
Metastasis;
Angiogenesis

Abstract As one of the malignant tumors with high mortality, liver cancer has the characteristics of early invasion and metastasis, and lacks effective treatment methods. Therefore, it is urgent to find new safe, efficient and low-toxic anti-liver cancer drugs. In this study, the evaluated mechanism of *Ecklonia cava* polyphenols (7PE, 7-phloro-eckol) as a potential active substance for inhibiting liver cancer metastasis (HepG2 cell) and angiogenesis (HUVEC cell). The results have shown that 7PE can inhibit the expression of hypoxia-inducible factors (HIF-1α) protein by regulating the PI3K/AKT/mTOR and Ras/MEK/ERK/MNK signaling pathways under hypoxic conditions in HepG2 cells, thereby blocking the secretion of vascular growth factor (VEGF) protein. At the same time, 7PE can significantly inhibit the conduction of AKT and MAPK signaling pathways in HUVEC by suppressing the activation of VEGFR-2 protein, which verifies the prediction of molecular docking. Therefore, the results of this study provide a research basis for 7PE to inhibit metastasis and angiogenesis of liver cancer cells, and provide a theoretical basis for the high-value utilization of *Ecklonia cava*.

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Peer review under responsibility of King Saud University.

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1. Introduction

Hepatocellular carcinoma (HCC), a serious threat to human health and life, is the common primary liver malignant tumor, with high morbidity and mortality, and the trend of younger patients (Liao et al., 2020). According to the statistics of the World Health Organization, there were approximately

https://doi.org/10.1016/j.arabjc.2021.103187
1878-5352

841,080 new cases and 781,630 deaths cases caused by liver cancer in 2018, ranking the sixth of the most common malignant tumor and the fourth of leading cause of cancer-related death in the world. (Bray et al., 2018). At present, sorafenib (Shen et al., 2018) and regorafenib (Jordi Bruix, 2017) are the standard drugs approved by the FDA for the treatment of advanced liver cancer. Among them, sorafenib has dual anti-tumor effects, which can act on tumor cells and tumor angiogenesis at the same time. Its effect was not only to directly inhibit the proliferation of tumor cells by blocking RAF/MEK/ERK signaling pathway mediated, but also to block tumor angiogenesis by inhibiting VEGFR and platelet-derived growth factor receptor (PDGFR), indirectly to inhibit the growth of tumor cells, thereby significantly improving the overall survival rate of patients. However, the drug may cause symptoms of drug resistance, low immunity and prognostic recurrence (Llovet et al., 2018; Zhang et al., 2021). In addition, studies have shown that natural products have the potential to be safe, highly effective, low-toxic and to reverse drug resistance (Lu et al., 2007; Ribeiro et al., 2020). Therefore, researchers hope to find a natural compound with low side effects, high efficiency and low toxicity in natural products.

Liver cancer causes high mortality (82%) (Siegel et al., 2020), due to its unlimited proliferation, invasion and metastasis ability. Among them, the unlimited proliferation ability of liver cancer will lead to the formation of solid tumors and consume a lot of nutrients and oxygen, which will lead to the formation of a hypoxic environment inside the tumor (Carmeliet and Jain, 2000; Teleanu et al., 2019). Under the stimulation of hypoxic environment, liver cancer cells will obtain living space and nutrients through invasion, metastasis and angiogenesis (Carmeliet and Jain, 2011). Studies have shown that tumor cells can promote the expression of hypoxia-inducible factors (HIF-1α), leading to the secretion of angiopoietin, matrix metalloproteinases (MMPs), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) under hypoxic conditions (Gera et al., 1999). VEGF is the downstream target of HIF-1α, which can bind to vascular endothelial growth factor receptors (VEGFR-2) to stimulate the growth, proliferation, migration and angiogenesis of vascular endothelial cells (Cho et al., 2020a; Ferrara et al., 2003). Therefore, inhibiting the activity of HIF-1α protein and the activation of VEGFR-2 protein are important means to effectively inhibit tumor angiogenesis.

The extreme environment in the ocean has led to the diversity and specificity of the structure of natural products from marine sources. These natural products provide a research source for finding effective natural active compounds. Seaweed is the most ubiquitous marine biological resource, which is widely distributed, large in yield, easy to obtain, and contains a large amount of active substance (Wali et al., 2019). Therefore, algae are becoming the research object of effective inhibitors for the prevention and treatment of cancer. In particular, marine brown algae and red algae have attracted great interest, due to their potential ability to produce various biologically active derivatives (Scieszka and Klewicka, 2019). Among them, phloroglucinol and its derivatives, which exist in brown algae, have attracted attention because of their good biological activity (Li et al., 2009; Montero et al., 2018). As we all know, brown algae is widely distributed on South Korea, Japan and China (Wijesinghe and Jeon, 2012). Among them, *Ecklonia cava*, an edible seaweed containing a large amount of phloroglucinol and its derivatives, was mainly used to produce food ingredients, animal feed, fertilizers and folk medicines (Kim et al., 2006). Moreover, it is worth pointing out that the bioavailability of phlorotannins was related to its molecular weight and lipophilicity (Bialonska et al., 2009; Corona et al., 2016). Excessive molecular weight leads to low bioavailability, and good lipophilic performance improves bioavailability. And the biological activity of phlorotannins was related to the number and position of hydroxyl groups. Therefore, within an appropriate molecular weight range, the greater the number of phenolic hydroxyl groups of polyphenols, the stronger the activity. 7PE of molecular weight was 281.36 Da, and have 8 hydroxyl groups, so has high research value. The compounds of *Ecklonia cava* show broad biological activity and therapeutic prospects, such as anti-oxidation (Kyoung Ah et al., 2005), anti-inflammatory (Cho et al., 2020b), anti-cancer (Kim et al., 2015), anti-neuroinflammatory (Lee et al., 2018), anti-diabetes (Park et al., 2018), and anti-allergic (Han et al., 2020), radiation protection (Piao et al., 2012). In addition, related reports have shown that *Ecklonia cava* phloroglucinol derivative (7PE, 7-phloro-eckol) has antioxidant activity (Li et al., 2009). But the anti-tumor angiogenesis mechanism has not been reported yet.

Therefore, we conducted the following research. Firstly, HepG2 cells and HUVEC were used to establish an invitro anti-tumor angiogenesis research model. Firstly, through MTT and scratch test to detect the effect of 7-phloro-eckol on the migration ability at non-cytotoxic concentration. Secondly, the effect of 7PE on the secretion of MMP-1, MMP-9, IL-8 and VEGF proteins, and the expression of HIF-1α protein in HepG2 cells under hypoxia was tested. Finally, revealing the interaction between VEGF receptors (VEGFR-2) and VEGF and its signal pathway is an effective way to inhibit angiogenesis in HUVEC.

The purpose of this study was to evaluate the inhibitory effect of 7PE on liver cancer cells and angiogenesis through in vitro experiments and molecular docking analysis, and to provide research data for 7PE as a potential natural active compound derived from seaweeds.

2. Material and methods

2.1. Chemicals and materials

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA (0.25%), and penicillin/streptomycin were purchased from Gibco (New York, USA). DAPI was purchased from Solarbio Science & Technology Co., Ltd (Beijing, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide (MTT), and $CoCl_2$ $6H_2O$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human VEGF-165 (8065), PI3K (4257), p-PI3K (17366), AKT (4691), p-AKT (4060), mTOR (2972), p-mTOR (2971), P70S6K (2708), p-P70S6K (9205), HIF-1α (3716), β-actin (4970) and secondary antibodies (anti-mouse, 7076; anti-rabbit, 7074) were provided by Cell Signaling Technology (CST, MA, USA). Ras (sc-63), p-Ras(sc-136481), MEK (sc-81504), p-MEK (sc-81503), MNK (sc-133107), JNK (sc-7345), p-JNK (sc-6254), p38 (sc-535), p-p38 (sc-166182), ERK (sc-94), p-ERK (sc-81492) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). VEGF (EHC108), MMP-1 (EHC134),

MMP-9 (EHC115), IL-1β (EHC002b), IL-6 (EHC007), IL-8 (EHC008), TNF-α (EHC103a), and PDGF (EHC181) ELISA kits were purchased from Neobioscience Technology Co., Ltd (Shenzhen, Guangdong, China). VEGFR-2 (E-EL-H1603c) ELISA kit was purchased from Elabscience Biotechnology Co., Ltd (Wuhan, Hubei, China). Matrigel was purchased from BD Biosciences (San Jose, CA, USA). All other chemicals and solvents were of analytical grade.

2.2. Cell culture

HepG2 cells and HUVEC were provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM, 10% FBS, 100 μg/mL streptomycin and 100 U/mL penicillin in a humidified incubator of 5% CO_2 at 37℃.

2.3. Cell viability assay

HepG2 cells were cultured in 96-well plates (1×10^5 cells/mL, 100 μL) for 24 h. The each well was changed to 100 μL fresh serum-free medium containing 7PE (10, 20, 50, and 100 μM) for 24 h. Then 100 μL MTT (1 mg/mL) was replaced to each well, and incubated for 3 h at 37℃. And 100 μL DMSO was replaced to dissolve the formazan crystals. Finally, the absorbance was measured using a microplate reader (BioTek, Winooski, VT, USA) at 570 nm.

2.4. Cell wound healing assay

HepG2 cells were cultured in 24-well plates (4×10^5 cells/mL, 500 μL) for 24 h. Cells were scratched using a sterile pipette tip, and then washed with PBS to remove cell debris. Then each well was changed to 500 μL fresh serum-free medium containing 7PE (10, 20, 50 and 100 μM) and incubated in CO_2 incubator for 12 or 24 h. The cell migration across injury line was observed using a microscope (Olympus, Tokyo, Japan) and recorded photographically.

2.5. Enzyme linked immunosorbent assay (ELISA)

Cells were treated with 7PE (10, 20, and 50 μM) for 24 h. Then conditional media or cells lysates were harvested and centrifuged (12000 rpm, 4℃, 10 min) to get the supernatants. The concentration of protein was analyzed according to the manufacturer's protocol.

2.6. Molecular docking

Use ChemDraw (PerkinElmer, Waltham, Mass, USA) to draw the chemical structure of 7PE (Fig. 1A). And then use Chem3D (PerkinElmer, Waltham, Mass, USA) to convert it into a 3D structure, and use the MMFF94 force field to optimize. The 3D structure of HIF-1α (PDB ID: 1H2N) and VEGFR-2 (PDB ID: 2OH4) can be downloaded from RCSB Protein Data Bank (www.rcsb.org). HIF-1α, VEGFR-2 and compound 7PE were converted to PDBQT grid using autodock tools (Scripps Research Institute, La Jolla, CA, USA). Using autodock vina (Scripps Research Institute, La Jolla, CA, USA) for molecular docking research. In order to increase the accuracy of the calculation, the parameter exhaustiveness was set to 100. Finally, the constellation with the highest score was selected to analyze the results using PyMoL (DeLano Scientific LLC, San Carlos, CA, USA) and Discovery Studio (Biovia, Waltham, Mass, USA).

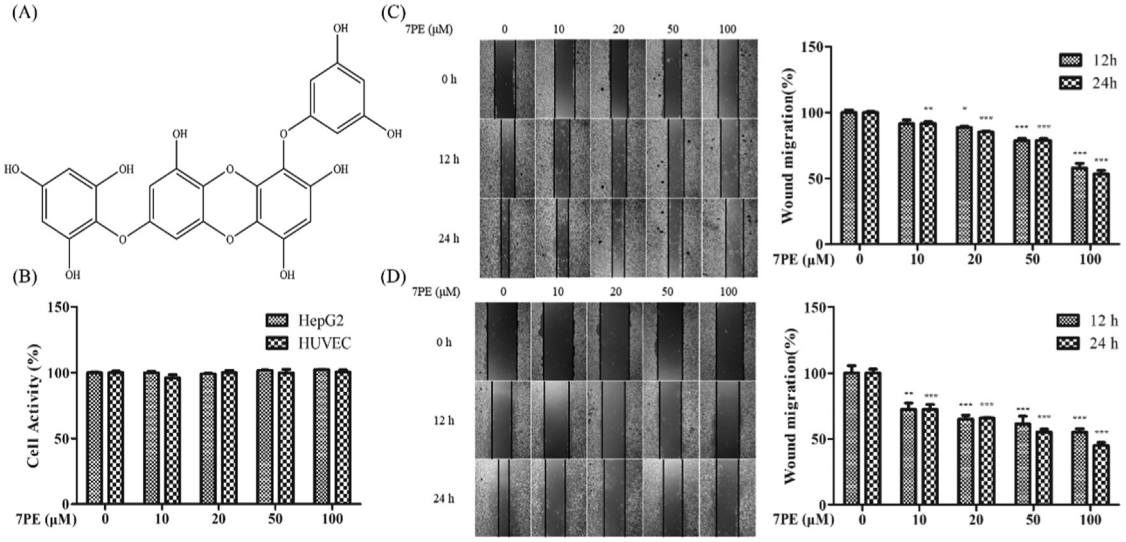

Fig. 1 (A) Chemical structure of 7-phloro-eckol (7PE) from the edible marine brown alga *Ecklonia cava*. (B) Cell viability of HepG2 cells and HUVEC after 24 h of treatment with 7PE by MTT method. And migration ability of HepG2 cells (C) and HUVEC (D) was tested by cell scratch test. After scratching the fused cell monolayer to produce scratch wounds, they were treated with 7PE, and then the migration was observed at 12 h and 24 h. Data are shown as mean ± SD (n = 3). # Compared with the blank group, $p < 0.05$. * Compared with the control group; * Compared with the control group, $p < 0.05$; ** Compared with the control group, $p < 0.01$; *** Compared with the control group, $p < 0.001$. Yang et al., 2021.

2.7. Western blot

HepG2 cells were cultured in 6-well plates (1×10^5 cells/mL, 1 mL) for 24 h. The each well was changed to 1 mL fresh serum-free medium containing 7PE (10, 20, 50, and 100 μM) for 2 h. Then each well was added 100 μM $CoCl_2$ or 10 ng/mL VEGF. Total cell protein was separated by using radio immunoprecipitation analysis (RIPA) buffer containing 1 mM PMSF. The BCA kit was used to quantify the protein content in lysates. An equal amount of protein was used for electrophoresis. The target protein was transferred to a nitrocellulose (NC) membrane by using SDS-PAGE. The membrane was visualized by blocking, incubating the primary antibodies, and performing secondary antibody incubation with an enhanced chemiluminescence (ECL) detection system (Syngene, Cambridge, UK). Image J (version 1.46r, NIH, Bethesda, Maryland, USA) was used to detect the band brightness in images and to export the data. β-actin was used as an internal control. Protein expression (relative to β-actin) was evaluated. The data were normalized with internal parameters and plotted using the GraphPad Prism5 software.

2.8. Immunocytochemistry

Cells were treated with 7PE (10 and 50 μM) and 100 μM $CoCl_2$ for 24 h, and then treated with 4% paraformaldehyde phosphate buffer solution (30 min, 4℃). Then, these cells were permeabilized with 0.2% Triton X-100 (20 min, 4℃) and blocked in 5% normal goat serum (1 h, RT), followed by incubation with anti-HIF-1α antibody (1:800, 4℃) overnight. Finally, the nucleus was stained with Actin-Red (KeyGEN Biotech, Jiangsu, China) and DAPI. The images were recorded by an inverted fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Tube formation assay

Cell suspension containing the 7PE (10 and 50 μM) was seeded in 96-well plates (NEST Biotechnology, Wuxi, China) pretreated with matrigel and incubated at 37℃. After 3 h, the level of tube formation was observed and photographed with an inverted microscope (Olympus, Tokyo, Japan).

2.10. Statistical analysis

All values are expressed as the mean ± standard deviation ($n = 3$). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) test with post hoc contrasts by Dunnett's test using GraphPad Prism 5.0. The statistical significance for all tests was set at $p < 0.05$.

3. Results

3.1. Effects of 7PE on the viability and migration ability of HepG2 cells and HUVEC

7-phloro eckol (7PE, Fig. 1A) was purified from marine edible brown algae *Ecklonia cava* (Li et al., 2009), and its molecular weight was 281.36 Da. At beginning of the study, we firstly determined the cytotoxicity of 7PE on HepG2 cells and HUVEC by the MTT assay. As depicted in Fig. 1B, there were no significant changed between 7PE-treated group and blank group, which indicated that 7PE is nontoxic to HepG2 cells and HUVEC. Thus, the employed concentrations (10, 20, 50, and 100 μM) of 7PE were used in the subsequent experiments.

Moreover, in the migration assay, untreated HepG2 cells and HUVEC apace migrated to the position of wound. On the contrary, 7PE treatment observably inhibited cell migration activity. For HepG2 cells, the inhibitory rate is 11.5–42.1% at 12 h and 14.7–46.5% at 24 h (Fig. 1C). And the inhibitory rate is 27.4–44.8% at 12 h and 27.5–54.9% at 24 h (Fig. 1D) on HUVEC. These dates indicated that 7PE can inhibit metastasis and invasion in HepG2 cells and HUVEC, which is not dependent concentrations on cytotoxicity.

3.2. Effects of 7PE on the metastasis and angiogenesis ability of HepG2 cells induced by hypoxia

3.2.1. Inhibition of 7PE on MMP-1, MMP-9, IL-8 and VEGF protein expression induced by hypoxia in HepG2 cells

Tumor cells can hydrolyze the surrounding matrix by secreting metal matrix proteases (MMPs), thereby causing invasion and metastasis (Gong et al., 2019). MMP-1 and MMP-9 are important members of the metal matrix protease family. And IL-8 and VEGF can promote tumor angiogenesis (Huang et al., 2016; Ju et al., 2017). In order to evaluate the invasion, metastasis and angiogenesis ability of 7PE, we measured the concentration of MMP-1, MMP-9, IL-8 and VEGF secreted by ELISA kit. As shown in Fig. 2A, 7PE treatment inhibited the release of MMP-1, MMP-9, IL-8 and VEGF protein in a concentration dependent manner.

3.2.2. Molecular docking predicts the interaction between HIF-1α protein and 7PE

HIF-1α is an important cytokine for tumor cell proliferation, metastasis and angiogenesis, and it can induce overexpression of VEGF. In order to elucidate the molecular mode of action of HIF-1α protein and compound 7PE. 7PE was docked with the active pockets of HIF-1α proteins to obtain the optimal docking structure. And the affinity of HIF-1α proteins was −8.5 kcal/mol. As Fig. 2B, the 7 phenolic hydroxyl groups of 7PE can form 8 hydrogen bonding. These hydrogen bonds are the main force between 7PE and HIF-1α, making 7PE and HIF-1α form a stable complex. Molecular docking simulation provides predictions for the interaction between 7PE and HIF-1α.

3.2.3. Effect of 7PE on HIF-1α expression induced by hypoxia in HepG2 cells

Western blot and immunocytochemistry were used to study the expression of HIF-1α protein. As shown in Fig. 2C and D, 7PE can effectively down-regulates the expression of HIF-1α protein in HepG2 cells. In Fig. 2D, DAPI and Actin stained the nucleus and cytoskeleton of HepG2 cells, respectively. We found that the DNA and cell structure of HepG2 cells were intact under hypoxic conditions. And after 7PE was added, the DNA was reduced and the cell structure was destroyed in HepG2 cells. Therefore, 7PE can inhibit the expression of hypoxia-inducible factor-1α protein, and affect the growth and state of HepG2 cells in a hypoxic environment.

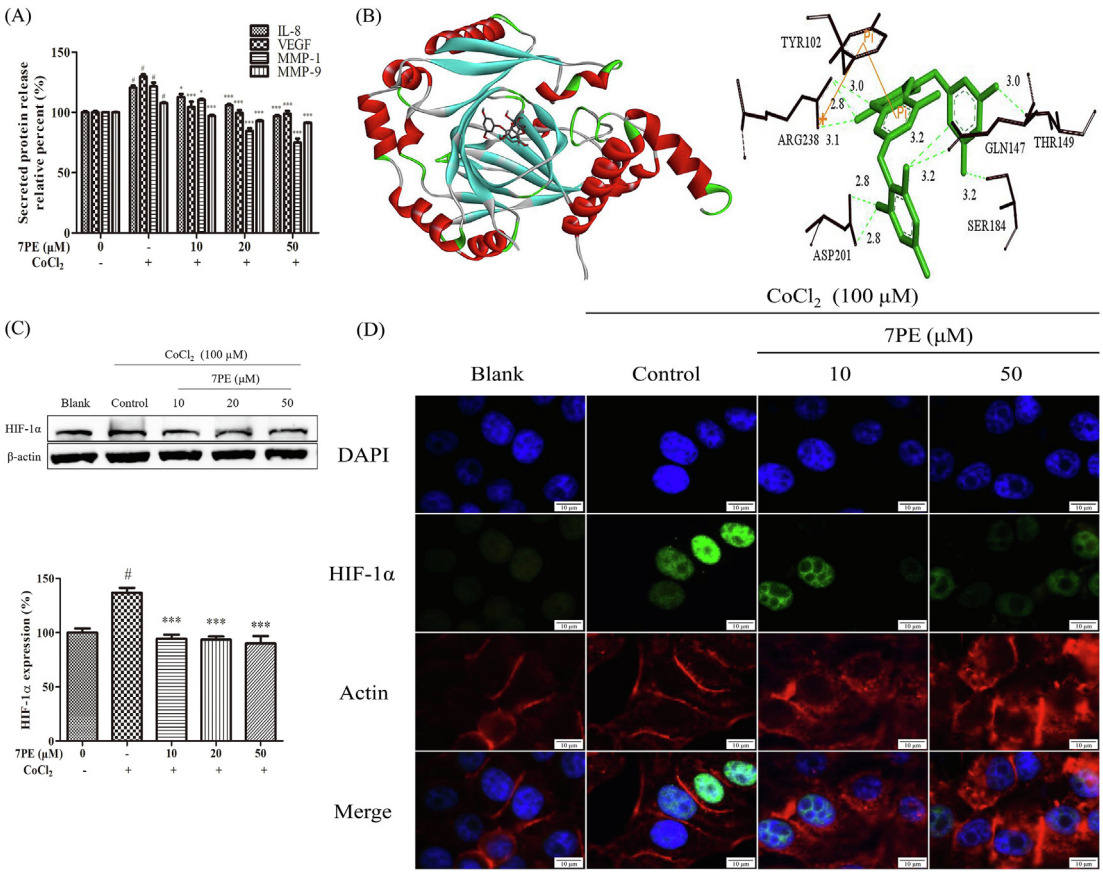

Fig. 2 (A) The release of IL-8, VEGF, MMP-1 and MMP-9 protein in the supernatant of HepG2 cells induced by $CoCl_2$ was determined by ELISA kits. (B) Molecular docking prediction obtains the best docking structure and the active site of 7PE and HIF-1α protein. (C) The expressions of HIF-1α proteins levels. β-actin was used as an internal control. Protein expression (relative to β-actin) was evaluated. (D) Immunofluorescence determination of HIF-1α protein. The nucleus was stained with DAPI, the cytoskeleton was stained with ActinRed, and the cells were immunostained with HIF-1α antibody. Images were recorded by an inverted fluorescence microscope. Data are shown as mean ± SD (n = 3). # Compared with the blank group, $p < 0.05$; * Compared with the control group; * Compared with the control group, $p < 0.05$; *** Compared with the control group, $p < 0.001$. Yang et al., 2021.

3.2.4. Effect of 7PE on PI3K/AKT/mTOR/P70S6K and Ras/MEK/ERK/MNK signaling pathway induced by hypoxia in HepG2 cells

Through western blot, this research found that the overexpression of HIF-1α protein can be caused by activating the PI3K/AKT/mTOR and Ras/ERK signaling pathways induced by hypoxia. However, the results have shown that 7PE can effectively down-regulate the expression of PI3K/AKT/mTOR/P70S6K (Fig. 3A) and Ras/MEK/ERK/MNK (Fig. 3B) protein in a dose-dependent manner.

From the above results, it can be seen that 7PE can block the expression of HIF-1α protein by inhibiting PI3K/AKT and Ras signal transduction pathways, thereby controlling the secretion of VEGF protein and achieving the effect of inhibiting tumor angiogenesis.

3.3. Effect of 7PE on the angiogenesis ability of HUVEC under VEGF stimulation

3.3.1. Molecular docking predicts the interaction between VEGFR-2 protein and 7PE

As a receptor on the surface of vascular endothelial cells, VEGFR-2 is the downstream target of VEGF. And VEGF can promote the proliferation, migration and angiogenesis of vascular endothelial cells by stimulating VEGFR-2 (Cho et al., 2020a). In this study, 7PE was docked with the active pockets of VEGFR-2 proteins to obtain the optimal docking structure. The affinity of VEGFR-2 proteins was −10.1 kcal/mol. In Fig. 4A, 7PE was surrounded by an active pocket composed of amino acids LEU838, VAL846, GLU883, VAL914, GLU915, CYS917, LYS918, GLY920 and PHE1045. Moreover, the 7 phenolic hydroxyl groups of 7PE can form 8 hydrogen bonds, which are the most important force between 7PE and VEGFR-2, and enables them to form a stable complex.

In addition, the above-mentioned molecular docking studies provide a reasonable basis for conjectures for the competition between 7PE and VEGF at the site of action of VEGFR-2.

3.3.2. Effect of 7PE on VEGFR-2, AKT/mTOR and MAPK signaling pathway induced by VEGF in HUVEC

Detection of cell lysate by ELISA have shown that 7PE intervention can significantly down-regulate the expression of VEGFR-2 and PI3K in HUVEC (Fig. 4B). At the same time, the results verified the results of molecular docking. In addition, we studied the expression of AKT/mTOR/P70S6K and

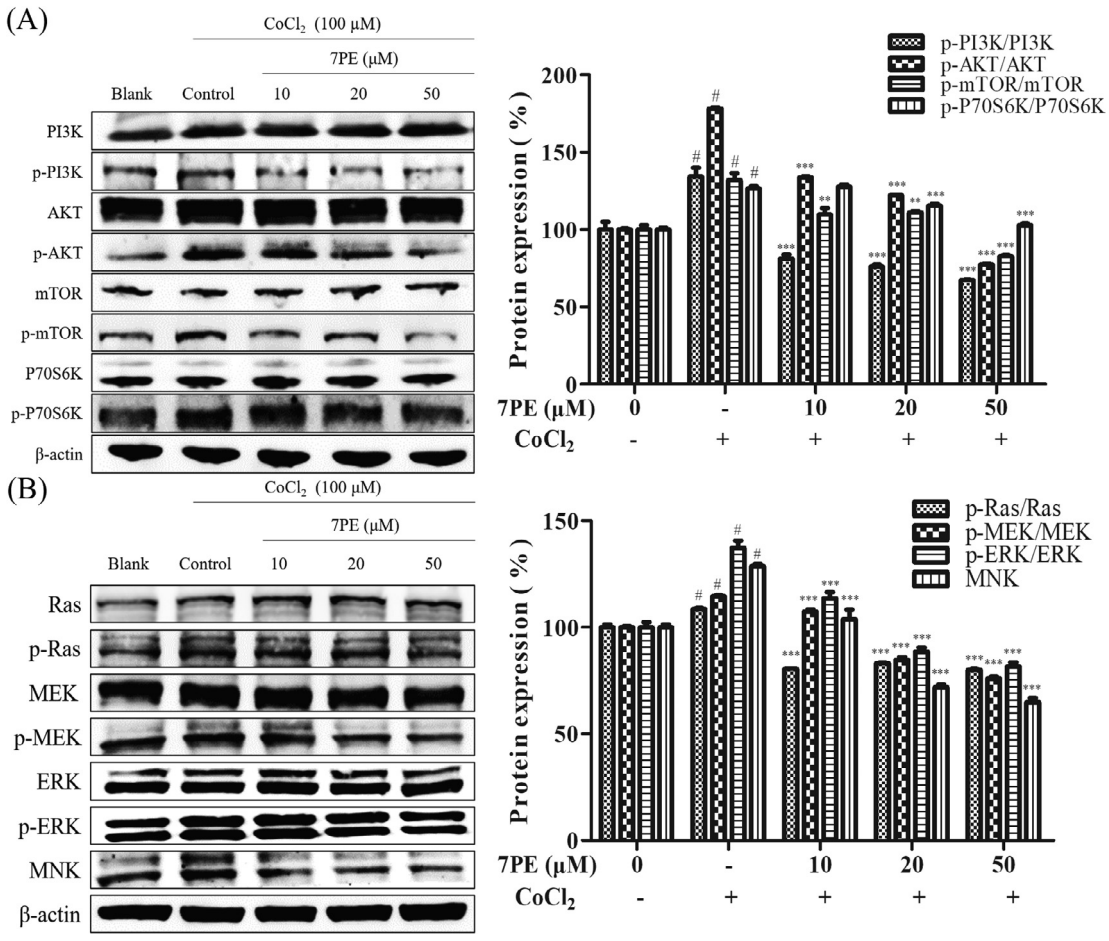

Fig. 3 Effect of 7PE on PI3K/AKT/mTOR and Ras/ERK signaling pathway proteins levels in $CoCl_2$-induced HepG2 cells. (A) The expressions of PI3K/AKT/mTOR signaling pathway proteins levels. (B) The expressions of Ras/ERK signaling pathway proteins levels. β-actin was used as an internal control. Data are shown as means ± SD (n = 3). # Compared with the blank group, $p < 0.05$. * Compared with the control group; ** compared with the control group, $p < 0.01$; *** compared with the control group, $p < 0.001$. Yang et al., 2021.

MAPK signals using western blot. And the results have shown that 7PE can reduce the expression of AKT/mTOR/P70S6K (Fig. 4C) and MAPK (Fig. 4D) signaling pathways in a dose-dependent manner.

From the above results, we found that 7PE prevents angiogenesis of HUVEC by blocking the activation of VEGFR-2 protein and inhibiting the AKT/mTOR and MAPK signaling pathways.

3.3.3. Inhibition of 7PE on IL-1β, IL-6, TNF-α and PDGF protein expression in HUVEC

HUVEC stimulated by VEGF produce inflammatory factors and PDGF, which will change the environment around vascular endothelial cells, and stimulate pericytes to produce VEGF, and further promote the angiogenesis process. In this study, ELSA was used to find that the inflammatory factors (IL-1β, IL-6, TNF-α) and PDGF (Fig. 4E) in the supernatant of HUVEC were significantly down-regulated under the intervention of 7PE. The above results indicate that the inflammatory factors and PDGF produced by VEGF stimulation in HUVEC can be inhibited by 7PE.

3.3.4. Effect of 7PE on the tube forming ability of HUVEC

Tube formation experiment is an important invitro model for testing angiogenesis activity of HUVEC. In this study, the blank group without 7PE treatment can form an obvious network structure, and adding 10 and 50 μM 7PE can significantly reduce the formation of tube structure (Fig. 4F). This result further directly proves that 7PE has the effect of reducing angiogenesis.

4. Discussion

As one of the well-known malignant tumors with high morbidity and mortality, liver cancer has problems such as high recurrence rate, easy metastasis of cancer cells, and poor prognosis (Ma et al., 2017). More and more studies have proved that the active ingredients of natural products overcome the drug resistance of tumor cells through a variety of molecular mechanisms, and have the role of preferentially killing cancer cells, with little side effects, resistance to drug resistance, and regulating the immunity of the body (Efferth et al., 2019; Khalifa et al., 2019; Kumar and Jaitak, 2019). Based on the above

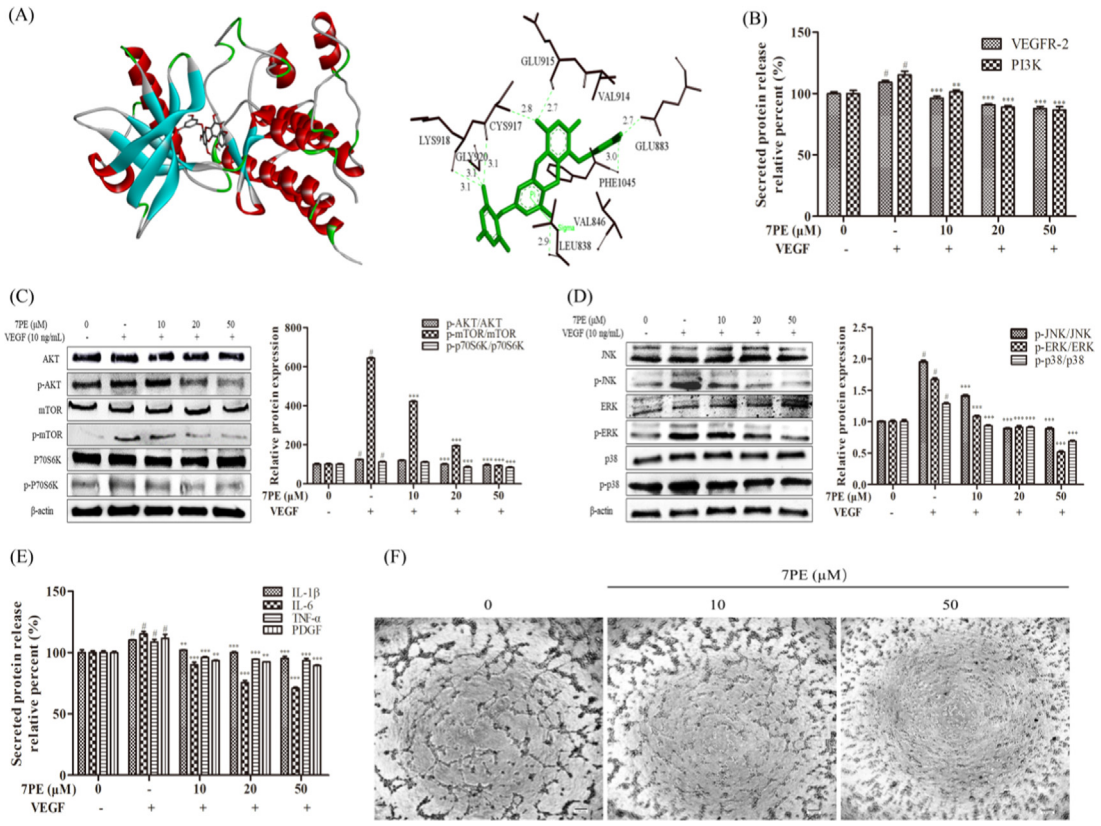

Fig. 4 (A) Molecular docking prediction obtains the best docking structure and the active site of 7PE and VEGFR-2 protein. (B) Determination of VEGFR-2 and PI3K protein level in VEGF-induced HUVEC. The cell lysates of HUVEC treated with 7PE was tested using an ELISA kit. (C) The expressions of AKT/mTOR signaling pathway proteins levels. (D) The expressions of MAPK signaling pathway proteins levels. β-actin was used as an internal control. (E) Determination of IL-1β, IL-6, TNF-α and PDGF protein release in VEGF-induced HUVEC. The supernatant of HUVEC treated with 7PE was tested by ELISA kit. (F) Effect of 7PE on tube formation of HUVEC. Data are shown as means ± SD (n = 3). # Compared with the blank group, $p < 0.05$. * Compared with the control group; ** compared with the control group, $p < 0.01$. *** compared with the control group, $p < 0.001$. Yang et al., 2021.

situation, this study used the natural phloroglucinol (7PE, 7-phloro-eckol) isolated from the edible brown algae *Ecklonia cava* in the previous study (Li et al., 2009), and for the first time studied the molecular mechanism of liver cancer invasion, metastasis and angiogenesis activity.

At present, the main methods known to control tumor angiogenesis include inhibiting tumor proliferation, invasion and metastasis, and controlling tumor cells to secrete VEGF and blocking its binding to receptors on vascular endothelial cells. Sadeeshkumar (Sadeeshkumar et al., 2017) and Lee (Lee et al., 2019) have shown that seaweed polyphenols (Dieckol) isolated from *Ecklonia cava* can inhibit tumor invasion and angiogenesis. In this study, MTT and cell scratch test were used to study the inhibitory effect of 7PE on the migration of HepG2 cells and HUVEC without cytotoxicity. The results have shown that when the concentration was less than 100 μM, the inhibitory effect of 7PE on migration was concentration-dependent. This indicates that 7PE may have the same ability as dieckol to inhibit invasion and migration, which provides a basis for further study of its mechanism.

Insufficient oxygen supply in the tumor microenvironment is related to the degree of tumor progression (Albini et al., 2012). This is because liver cancer needs to rely on the formation of new blood vessels in the process of forming solid tumors to provide necessary nutrients and oxygen. In this process, a large number of cytokines are required to participate. Among them, MMP-1 (Bower et al., 2004) and MMP-9 (Cheng et al., 2006; Yu and Lee, 2016) are important members of the metal matrix proteases (MMPs), which are related to the invasion ability of liver cancer, and are the signs of tumor invasion and metastasis. VEGF is a secreted protein that can act as an effective mitogen for vascular endothelial cells, and is one of the main regulators of angiogenesis and neovascularization (Carbajo Pescador et al., 2013). In this experiment, we found that 7PE can significantly reduce the secretion of MMP-1/9 and VEGF protein in HepG2 cells under hypoxic conditions (Fig. 2A), which determined that 7PE has the effect of inhibiting invasion and metastasis.

As we all know, in a hypoxic environment, HIF-1α is considered to be a key protein that can effectively regulate the production of angiogenesis-related cytokines. HIF-1α can directly regulate oxygen homeostasis. Under normoxic condition, oxygen can cause HIF-1α ubiquitination and subsequent degradation; while under hypoxic condition, HIF-1α can be stabilized and translocated to the nucleus, and induce the production of MMP-1/9 and VEGF protein (Gupta et al., 2013). Therefore,

it can be seen from the analysis of Fig. 2 that under hypoxic conditions, 7PE can effectively down-regulate the expression of MMP-1/9 and VEGF protein by reducing the expression and activity of HIF-1α protein, thereby inhibiting tumor growth and angiogenesis.

In addition, the current research on tumor angiogenesis is mainly focused on the expression of HIF-1α protein and the secretion of VEGF (Liao et al., 2020; Teleanu et al., 2019). Therefore, we used molecular docking to predict the possible interaction between 7PE and HIF-1α protein. However, these studies have not conducted in-depth studies on the signaling pathways of HIF-1α protein expression. As shown in Fig. 3, this study found that through western blotting that there are two signaling pathways that affect the expression of HIF-1α protein in HepG2 cells. These two pathways are PI3K/AKT/mTOR/P70S6K and RAS/MEK/ERK/MNK, and 7PE can significantly reduce the overexpression of related protein phosphorylation caused by hypoxia. Among them, the PI3K/AKT signaling pathway plays an important role in regulating cell growth, migration, invasion, transcription and protein synthesis. The Ras/ERK signaling pathway plays a role in cell growth, differentiation and transcription. These results have shown that 7PE can indirectly regulate the expression of HIF-1α protein and further regulate the secretion of VEGF protein through the above two signal pathways, thereby effectively controlling the process of tumor angiogenesis.

In the study of tumor angiogenesis, HUVEC is a good research model. The cell surface has a VEGF receptor, VEGFR-2, which is an important transmitter that effectively receives VEGF signals and transmits them to cells to accelerate the rapid proliferation, invasion and growth of vascular endothelial cells (Cerezo et al., 2017). Interestingly, the molecular docking of 7PE and VEGFR-2 protein found that 7PE can binds tightly to the active site of the protein, and the active site was consistent with the previously reported inhibitor binding site (Hasegawa et al., 2007), which indicates that 7PE may become a VEGFR-2 inhibitor. VEGFR-2 can be activated by VEGF to activate the downstream PI3K/AKT/mTOR/P70S6K and MAPK signaling pathways to promote the proliferation, invasion and angiogenesis of vascular endothelial cells (Chin et al., 2018; Zhang et al., 2017). This study also confirmed this point by ELISA and western blotting results. As shown in Fig. 4B, C and D, AKT/mTOR and MAPK-related protein phosphorylation levels were significantly increased under VEGF stimulation, but 7PE effectively contained this process. Among them, 7PE has a particularly obvious inhibitory effect on mTOR, JNK and ERK proteins. mTOR protein has the function of regulating cell growth, survival and proliferation, and plays a role in tumor angiogenesis (Laplante and Sabatini, 2012). ERK and JNK play a role in cell growth, differentiation, transcription and migration (Chang and Karin, 2001). In addition, HUVEC treated with VEGF will cause inflammation to produce inflammatory factors (IL-1β, IL-6, TNF-α), and overexpress PDGF. PDGF can recruit pericytes and activate PDGF receptors on the surface of pericytes to secrete VEGF, thereby further promoting angiogenesis. However, ELISA results illustrated that 7PE can effectively inhibit this process. Finally, the tube formation results further confirmed the effect of 7PE in inhibiting angiogenesis.

Moreover, the prediction results of molecular docking demonstrated that the scores of HIF-1 and VEGFR-2 were −8.5 and −10.1 kcal/mol, respectively, which signified that 7PE bind to VEGFR-2 more easily. According to the analysis, 7PE may not directly bind to HIF-1α protein, but may indirectly control its expression by acting on the upstream protein of HIF-1 protein. On the contrary, 7PE may compete with VEGF for binding sites and directly bind to VEGFR-2, suppressing the expression of VEGFR-2 protein. This provides research directions for subsequent angiogenesis research.

Combined with the above research results, it is revealed that 7PE can act on HepG2 cells and HUVEC at the same time, and has dual anti-tumor effects. 7PE can not only inhibit the expression of HIF-1α protein and the secretion of VEGF protein by blocking PI3K/AKT/mTOR/P70S6K and RAS/MEK/ERK/MNK mediated signal transduction pathways on HepG2 cells, but also effectively inhibit the binding of VEGF and VEGFR-2 and the secretion of PDGF on

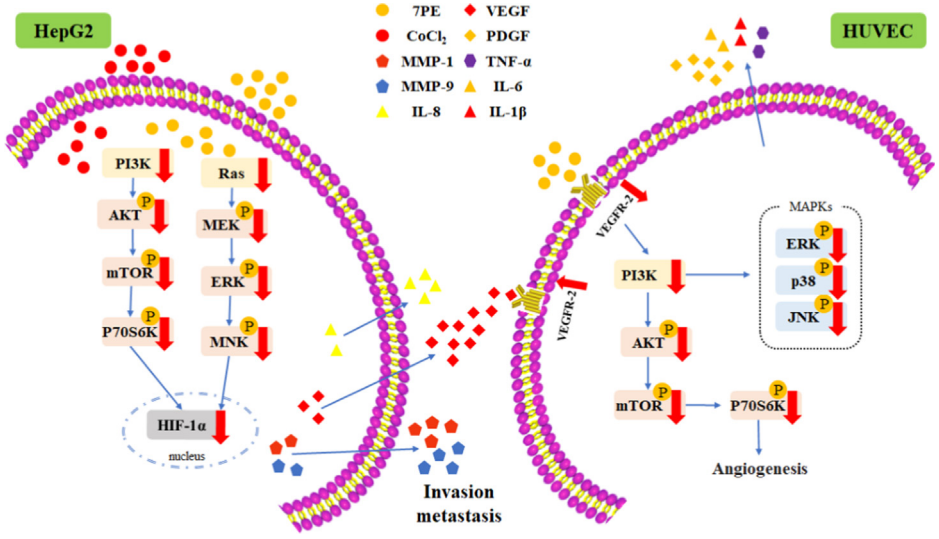

Fig. 5 Main signal pathway of 7PE inhibiting metastasis and angiogenesis in HepG2 cells and HUVEC. Yang et al., 2021.

HUVEC, thereby blocking tumor angiogenesis. In addition, the biological activity of polyphenols was in direct relation with their chemical structures, such as the number and position of hydroxyl groups (Singh et al., 2017). The greater the number of hydroxyl groups, the stronger the antioxidant activity of polyphenols, but as the number of hydroxyl groups increases, the stability of polyphenols will decrease. Moreover, from the perspective of molecular docking, the hydroxyl structure in polyphenols can form a hydrogen bond with the target protein (Lin et al., 2021). Therefore, the number and position of hydroxyl groups are important indicators for studying the biological activity of polyphenols. At present, in the research on polyphenol compounds of *Ecklonia cava*, the two structures of eckol and dieckol were relatively mature, and both compounds have good antioxidant and anti-tumor activities (Kang et al., 2013; Kyoung Ah et al., 2005; Lee et al., 2019). According to research, eckol and dieckol have 6 and 11 hydroxyl groups, respectively, while 7PE has 8 hydroxyl groups. Therefore, 7PE with a hydroxyl number between eckol and dieckol may have better performance in terms of biological activity and structural stability. Therefore, we have reason to believe that 7PE is a brown algae polyphenol with good anti-tumor angiogenesis activity isolated from the edible algae *Ecklonia cava*. In addition, the research model (Fig. 5) shows that anti-tumor angiogenesis inhibitors targeting tumor cells and vascular endothelial cells may be attracting attention.

5. Conclusions

This research reveals for the first time from the molecular level that the brown algae polyphenol 7PE derived from *Ecklonia cava* effectively inhibits tumor angiogenesis in HepG2 cells and HUVEC. The mechanism is mainly through PI3K/AKT/mTOR and Ras/ERK signaling pathways to inhibit the expression of HIF-1α protein to block the production of VEGF protein, and then block the activation of VEGFR-2 signaling pathway, thereby effectively controlling the process of tumor angiogenesis. Therefore, at the cellular level, 7PE has the biological activity of inhibiting the metastasis and angiogenesis of HepG2 cells.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The research was funded by the Natural Science Foundation of Guangdong Province (2020A1515011075) and the Yangfan Scarce Top Talent Project of Guangdong Province (201433009). The research was supported by the 2020 Shenzhen International Scientific and Technological Cooperation R&D Project, support was provided by Guangdong Tongde Pharmaceutical Co., Ltd and funded by the Development Project about Marine Economy Demonstration of Zhanjiang City (2017C8B1). In addition, the research was funded by the Fund of Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang, ZJW-2019-07).

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