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Effect of Oroxyllum indicum on hepatocellular carcinoma via the P53 and VEGF pathways based on microfluidic chips

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Abstract

Background Hepatocellular carcinoma (HCC), abbreviated as liver cancer, is one of the most common cancers in clinics. HCC has a wider spread and higher incidence due to its high malignancy and metastasis. In HCC, effective strategies to block cancer cell migration, invasion, and neovascularization need to be further studied. Consumption of flavonoid-rich Oroxyllum indicum (OI) has been associated with multiple beneficial effects, including anti-inflammatory and anticancer properties, but the potential effects on HCC have not been thoroughly investigated.

Objective In this study, we aimed to reveal the effect of OI on HCC and its potential mechanism through microfluidic technology.

Methods We designed microfluidic chips for cell migration, invasion, and neovascularization to evaluate the effect of OI on HepG2 cells. To further explore the mechanism of its anti-liver cancer action, the relevant signaling pathways were studied by microfluidic chips, RT-qPCR, and immunofluorescence techniques. Compared to the control group, cell migration, invasion, and angiogenesis were significantly reduced in each administration group. According to the P53 and VEGF pathways predicted by network pharmacology, RT-qPCR and immunofluorescence staining experiments were conducted.

Results The results showed that OI upregulated the expression of *Bax*, *P53* and *Caspase-3* and downregulated the expression of *Bcl-2* and *MDM2*. It has been speculated that OI may directly or indirectly induce apoptosis of HepG2 cells by regulating apoptosis-related genes. OI blocks the VEGF signaling pathway by downregulating the expression levels of *VEGF*, *HIF-1 α* and *FGFR* and inhibits the migration and invasion of HepG2 cells and the formation of new blood vessels.

Conclusion Our findings suggest that OI may inhibit the migration, invasion, and neovascularization of HepG2 cells, and its regulatory mechanism may be related to the regulation of the P53 and VEGF pathways.

Highlights

- Oroxyllum indicum has a therapeutic effect on liver cancer.
- Oroxyllum indicum reduced the migration and invasion of HepG2 cells.

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- Oroxylinum indicum inhibited neovascularization in HepG2 cells.
- Oroxylinum indicum activated the P53 pathway and blocked the VEGF pathway.

Keywords Oroxylinum indicum, Microfluidic chip, Anti-liver cancer, HUVEC, HepG2

Introduction

HCC is one of the leading causes of cancer-related death in many parts of the world [1, 2]. Significant progress has been made in the prevention, diagnosis and treatment of HCC. However, more than 50% of HCC patients are diagnosed with advanced stage disease [3, 4]. Although cirrhosis underlies the majority of HCC cases, many molecular pathways have been implicated in HCC carcinogenesis, including the P53, Akt/mTOR, VEGFR and EGFR/RAS/MAPK pathways.

Determining the optimal treatment plan takes into account not only the burden and extent of HCC but also the patient's performance status, underlying liver function, extrahepatic disease, and comorbidities. Radiofrequency or microwave ablation, hepatectomy or liver transplantation, all potential therapeutic options for HCC, are radical therapies. For patients who are not candidates for radical treatment, local regional treatments such as transarterial chemoembolization, transarterial radioembolization, and stereotactic body radiation can improve survival and quality of life (QOL). Sorafenib, a multikinase VEGF inhibitor, is the most widely used systemic chemotherapy agent. Unfortunately, the development of resistance to sorafenib is becoming more common [5]. Sorafenib is approved as a first-line agent for the treatment of unresectable, advanced HCC, but there are some side effects that affect patients' QOL [6, 7]. However, traditional Chinese medicine (TCM) has obvious efficacy and fewer side effects, which can improve the QOL of patients with symptoms [8–10].

Oroxylinum indicum (OI) is an herbal medicine belonging to Bignoniaceae that constitutes flavonoids, namely, Oroxylin A, Baicalin, and Chrysin. OI has a wide range of pharmacological activities, so parts of the plant have been used to treat a variety of diseases, such as cancer, fever, hepatitis, and diarrhea [11]. OI has anticancer, antibacterial, anti-inflammatory and other effects. Some of these actions are direct and some are indirect, and each mechanism must be clearly understood to improve the plant's efficacy in treating disease. However, the mechanism of the pharmacological action of this natural product remains unclear. Therefore, more studies are needed to verify the mechanism of action of OI as a potential treatment for a variety of human diseases [12]. Studies have reported that OI has a certain preventive and therapeutic effect on animal models of liver cancer, but there are few studies on its therapeutic mechanism [13,

14]. Although animal testing is essential for preclinical screening in drug discovery, there are limitations, such as ethical considerations and species differences. To avoid these problems, cell-based analysis using human cells has been actively promoted as an alternative [15, 16].

In this study, network pharmacology was used to screen the relevant targets and pathways. ELISA, RT-qPCR and immunofluorescence methods were used to study the relevant signaling pathways based on multimicrofluidic chips. The results of the designed microfluidic chip, RT-qPCR and immunofluorescence showed that OI inhibited the migration, invasion and neovascularization of HepG2 cells via the P53 and VEGF pathways.

Materials and methods

Instrument

The usage of a carbon dioxide (CO₂) incubator (SIM Corporation) was implemented for the purpose of cell culture, serving as a facilitator for optimal growth of cells in terms of both CO₂ and temperature.

The ECLIPSE Ti Research Grade Fluorescent Inverted Microscope (Nikon, Japan) exhibits remarkable optical clarity, thereby facilitating highly efficient cell culture observation and documentation.

Utilizing an Enzyme Label Instrument (Swiss TECAN company), the absorbance value of each well was measured within the ELISA reaction process, thereby enabling accurate analysis of the results.

Database

The Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (<http://tcmspw.com/tcmsp.php>) is widely acknowledged as a premier resource for the advancement of research in Chinese herbal medicine, offering a comprehensive elucidation of the intricate relationships between drugs, targets, and diseases.

The DisGeNET database (<https://www.disgenet.org/>) serves as a valuable platform by housing a vast collection of genes associated with human disease and variation accessible to the public. With its extensive and original indicators, DisGeNET facilitates the prioritization of genotype-phenotype relationships, offering a unique perspective.

OMIM (www.omim.org) is a comprehensive database that collates and arranges information pertaining to the human genome and its genetic manifestations.

STRING (<https://string-db.org/>), a database that collates known and predicted protein–protein interactions (PPIs), incorporates a variety of bioinformatic techniques to consolidate data and has been utilized in scientific research [17].

We utilized the Gene Ontology (GO) database (<https://geneontology.org/>) to delineate potential biological mechanisms behind therapeutic targets by identifying significant terms in the biological process (BP), cell component (CC), and molecular function (MF) categories. We used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.kegg.jp/>) to further explore the significant biological implications of therapeutic targets and validate the accuracy of the integrated results [18].

Cells and cell culture conditions

HepG2 cells (Shanghai Biotechnology Co., LTD.) were cultured in DMEM containing 10% fetal bovine serum (containing 100 U·mL⁻¹ penicilliomycin) and continuously cultured in an incubator at 37 °C and 5% CO₂. The cells grew to approximately 90% confluence and were digested with 0.25% trypsin. Cells at the logarithmic growth stage were inoculated in the chip used for the follow-up experimental study.

HUVECs (Shanghai Biotechnology Co., LTD.) were cultured in ECM (Saibikang Biotechnology Co., LTD.) containing 5% fetal bovine serum, 1% endothelial growth factor, and 1% cyanin-streptomycin and placed in a 5% CO₂ incubator at 37 °C. The solution was changed every other day. Cell growth to 80%~90% density passage. The 4~7 generation cells of logarithmic growth stage were selected for follow-up experimental study.

Plant materials and preparation of *Oroxylum indicum* extracts

Oroxylum indicum (specimen No.: 20200502) was collected from Datan Quanjian Chinese Herbal Medicine Decoration Co., LTD. and was identified as *Oroxylum indicum* (L.) Vent. dried ripe seeds by Professor Hui Zhang from Liaoning University of Traditional Chinese Medicine. In the initial research of the laboratory, a process for extraction and purification was identified as the best approach. The *Oroxylum indicum* was extracted three times using a 60% ethanol solution with a concentration of 15 times for 1 h. HPD-100 resin was utilized, and a sample concentration of 0.1 g·mL⁻¹ was employed with the crude drug BV column. Following the adsorption procedure, the impurities were eliminated by using 5 BV water followed by elution with 10 BV 60% ethanol. The purity level attained was no less than 90% in the final product [19].

Fabrication of microfluidic chips

Positive mold

First, the high-purity single-crystal single-cast silicon wafer was immersed in piranha wash solution (concentrated sulfuric acid:hydrogen peroxide=3:1) overnight to remove impurities. Then, it was cleaned by acetone and absolute ethanol ultrasonication for 15 min and by deionized water ultrasonic cleaning three times for 15 min each time. After cleaning, it was blown dry with a nitrogen generator and heated at 105 °C for 30 min to completely dehydrate it.

The SU-8 negative photoresist was naturally poured on the silicon wafer, and the photoresist was spin-coated evenly using a homogenizer [20] and then cured at 65 °C for 15 min, 95 °C for 30 min, and 65 °C for 15 min using a programmed heating table and cooled to room temperature. The photolithography mask was covered on the silicon wafer, placed on the photolithography machine, and exposed to UV light for 3 min. After that, it was transferred to the drying table and heated at 65 °C for 10 min, 90 °C for 30 min, and 65 °C for 10 min and then cooled to room temperature. The silicon chip was placed in the developing solution and shaken evenly to remove the cross-links on the surface of the silicon chip [21], and the residual developing solution was washed out with 75% ethanol. Finally, the silicon chip was heated at 120 °C for 30 min until the microchannel was solid and hard, and the positive mode of the chip was obtained.

PDMS channel layer

PDMS and curing agent were mixed and stirred at a ratio of 10:1 until a large number of fine bubbles were produced and degassed with a vacuum pump for 60 to 90 min until the bubbles disappeared completely. After that, it was poured on the positive mold of the chip and heated according to the heating program of 65 °C for 10 min, 95 °C for 30 min, and 65 °C for 10 min. After cooling to room temperature, holes were punched to obtain channels.

The bonding process

The glass sheet and the channel layer of the PDMS polymer to remove the surface dust were both put into the plasma cleaning machine using a high power gear (720 V/25 mA/18 W) depending on the size of the PDMS oxygen plasma excitation for 3~5 min. The PDMS polymer channel side down on the glass sheet vertically drove away the bubbles between the two layers and heated at 90 °C for 2 h [22].

The instructions of microfluidic chips

Cell migration chip

The chip is composed of a microvalve control layer, a fluid channel layer, and a glass substrate layer. The fluid channel layer can simultaneously screen a variety of anti-migration drugs of liver cancer cells, reflecting the integration and high efficiency of the microfluidic chip. The microvalve control layer is divided into a scratch simulation area and module unit connection area. The formation process of the scratch is as follows: after continuous liquid is pumped into the scratch simulation area, cells are injected from the cell entrance, and the cells cannot enter the scratch area due to the liquid pressure. After the cells adhered to the wall, the fluid in the scratch area was withdrawn, and the number of cells migrating to the scratch area was observed. The design drawing and photograph are shown in Fig. 1. The chip exhibits a more realistic simulation of a "scratch" by utilizing a valve-controlled device to continuously pump in and out liquid, which avoids physical damage to the cells. The cell culture area is equipped with cylindrical pillar structures to

provide support for the channel layer and prevent it from collapsing. The chip consists of four identical structural units, which allows for the simultaneous screening of multiple drugs.

Cell invasion chip

The cell invasion chip was composed of an upper channel layer, a polycarbonate membrane, a lower channel layer and a glass substrate layer from top to bottom. The polycarbonate membrane was adsorbed together by electrostatic adsorption, and the upper and lower channel layers were connected together by reversible hot bonds. The design concept of the chip comes from the Transwell chamber. Matrix gel is injected into the upper channel layer to simulate the three-dimensional cell survival environment, and the invasion activity of cells under the chemotaxis of the upper and lower layers is observed after the drug is introduced. The design drawing and photograph are shown in Fig. 2. This chip employs a unique technique wherein electrostatic attraction holds the polycarbonate film together, providing a secure connection

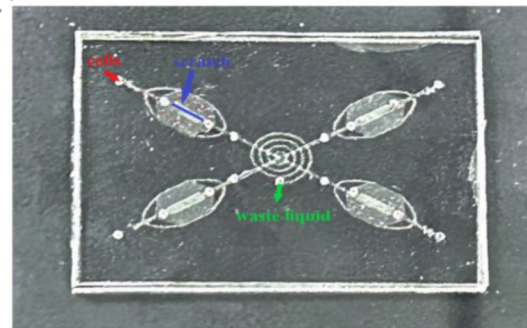
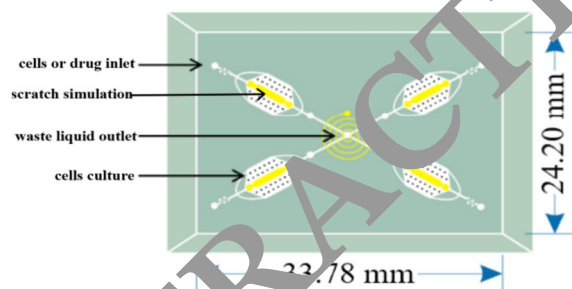


Fig. 1 Cell migration chip The fluid channel layer has four identical modules, which can simultaneously screen a variety of anti-migration drugs of liver cancer cells, reflecting the integration and high efficiency of the microfluidic chip

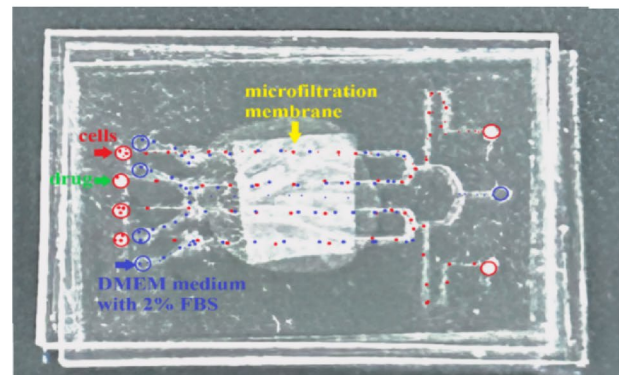
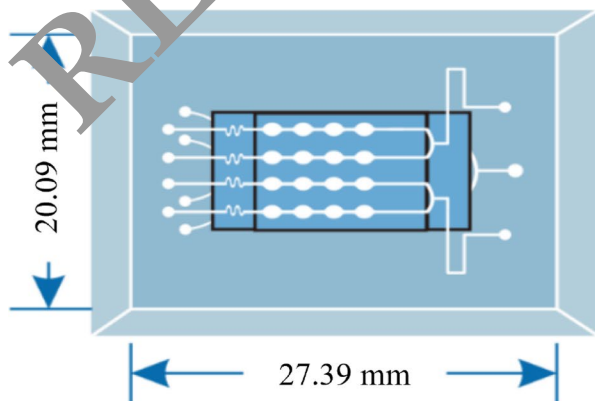


Fig. 2 Cell invasion chip Cancer cells can penetrate human diaphragm growth, so this experiment adopts the descent of the chip of the polycarbonate membrane simulation of the artificial membrane to conduct research on OI HepG2 cell activity. Microfluidic chips can control the fluid in time and space at the same time and better simulate the real environment in vivo

Fig. 3 Cell neovascularization chips Channel 1 and channel 5 are the culture medium channels that can not only ensure the supply of nutrients but also reduce the impact of fluid flow on cells. Passage between the small rectangular structure can guarantee the precise localization of fluid infusion, can not only realize physical isolation of different kinds of cells, and can realize the mutual communication between cells, conducive to control the position of the gel distribution at the same time, in ensuring that the channel can be completely filled with liquid limit contents as needed in the channel at the same time, not easy to overflow. The matrix collagen solution was injected into channel 3 to fill the channel and act as a barrier to separate the two types of cells. HepG2 cells were encapsulated by Matrigel and injected into channel 2 to establish a 3D growth condition, and HUVECs were distributed in channel 4 as a 2D monolayer of adherent growth

The assay based on cell invasion chip

The starved cells were slowly injected into the upper channel layer at a density of 2.5×10^5 cells/mL, and the lower channel layer was injected with DMEM containing 2% FBS to promote the adherence of the cells in the upper channel layer. After 12 h, the drug-containing culture medium was injected into the upper channel at a rate of $0.08 \mu\text{L} \cdot \text{min}^{-1}$, and the 10% FBS DMEM culture medium was injected into the lower channel at a rate of $0.1 \mu\text{L} \cdot \text{min}^{-1}$. After 24 h, the upper PDMS layer was removed, and a cotton swab was used to wipe the microporous membrane to filter the residual matrix and cells on the upper side. The microporous filter membrane was removed and stained with 0.1% crystal violet at room temperature in the dark. The decolorization solution was used to read the OD value of each group at 570 nm. The data and treatment results were analyzed. Relative rate (%) = (OD dosing/OD control) $\times 100$.

Effect of OI on the neovascularization of HepG2 cells

Perfusion and culture of HepG2 cells and HUVECs

HepG2 cells in the logarithmic growth phase were mixed with the prepolymer solution of matrix gel dissolved in advance, and the cell suspension was infused into the corresponding channel in the cell neovascularization chip at a cell density of 5×10^6 cells/mL to ensure that it was completely filled with the channel and did not spill into other channels. The perfused chip was incubated in a 37°C incubator. HUVECs in the logarithmic growth phase were selected and perfused into the corresponding channels in the chip at a cell density of 2×10^7 cells/mL.

The matrix collagen solution was first injected into channel 3 to fill the tube and incubated for 1 h to completely polymerize. Second, HepG2 cells in channel 4 and HUVECs in channel 5 were incubated in a cell incubator for 3 h until the endothelial cells adhered to the wall and the HepG2 cells were fully embedded in Matrigel. Finally, serum-free ECM culture medium was used to clean channel 3 and wash away the adherent cells. At the same time, channel 4 and channel 5 were perfused with serum-free ECM medium to provide nutrients for the cells.

Tube formation assay

Before the experiment, the 96-well plate was precooled at -20°C , and the Matrigel matrix gel was melted from -20°C to 4°C . The Matrigel gel was diluted 1 time with low serum medium to $60 \mu\text{L}$ per well and then incubated in a CO_2 incubator for 1 h.

The cells were divided into a control group, VEGF₁₆₅ group, sorafenib group, and low-, medium- and high-dose OI groups. HUVECs were digested to 2×10^5 cells/mL, the cell suspension was mixed with drug-containing

medium at an equal volume of 1:1, and the plates were plated at $200 \mu\text{L}/\text{well}$. After culturing for 6 h at 37°C in a 5% CO_2 incubator, the formation of microtubules was observed under an inverted microscope. Image software was used for image processing. The background was removed, the image was adjusted to black and white properties, and the number of linear structures with side lengths greater than $30 \mu\text{m}$ was counted to calculate the proportion of cavity area.

ELISA

A human VEGF ELISA kit (Sinovac, Shanghai, China) and a human HIF-1 α ELISA kit (Sinovac, Shanghai, China) were used to measure the levels of VEGF and HIF-1 α in the supernatants of medicated culture medium-treated HepG2 cells, according to the supplied instructions.

Molecular mechanism prediction

Acquisition of potential targets for active ingredients.

A comprehensive and effective collection of ingredients was meticulously curated from reliable sources such as the TCMSP (<http://tcmsp.com/tcmsp.php>). Utilizing the screening criteria of $\text{OB} \geq 30\%$ and $\text{DL} \geq 0.18$ [23].

Network construction

We entered 'hepatocellular carcinoma' in the DisGeNET and OMIM databases and obtained the anti-liver cancer target after retrieval, weight removal and summary. Target genes involved in active components were imported into the STRING database to obtain protein interaction data information, and protein interaction parameters with the highest confidence score > 0.9 were selected to construct the protein-protein interaction (PPI) network. The GO function (cell function, molecular function, and biological function) analysis and KEGG pathway enrichment [24–26] of coaction targets were conducted. $P < 0.05$ indicates significant differences.

RT-qPCR analysis

The chip for RT-qPCR analysis.

The chip is composed of a PDMS fluid channel layer and a glass substrate layer. The chip included a cell inlet, drug inlet, cell culture area and cell metabolite collection port. The design drawing and photograph are shown in Fig. 4. Our multifunctional chip boasts a unique ability to perform cell culture, drug stimulation, and cell sample collection, all in one compact device, which means that conventional culture containers such as culture flasks are no longer necessary. The chip is engineered to perfectly mimic the physiological environment of human cells, resulting in more precise and accurate detection outcomes.

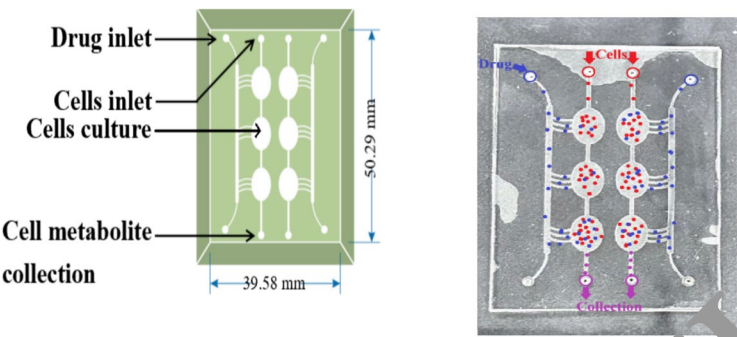


Fig. 4 The chip for RT-qPCR analysis The cell culture area can meet the number of sample cells used in the experiment. Cultured cells in the chip can make up for the traditional orifice or cultivation bottles, and other containers cannot be simulated in vivo. Building similar to the cells in the human body physiological micro space can further increase the reliability of the test results

Table 1 RT-qPCR system

Components	Volume
cDNA	2 μL
Forward Primer(10 μM)	1 μL
Reverse Primer(10 μM)	1 μL
2×TransStart Top Green qPCR SuperMix	10 μL
ddH ₂ O	6 μL
Total Volume	20 μL

The extraction of total RNA

HepG2 cells at the logarithmic growth stage were inoculated in the chip at an adjusted density of 8×10^6 cells/mL for dynamic culture. When the cells adhered to the wall grew to approximately 5%, the injection of culture medium was stopped, and different drugs and drug efficacy sites of different concentrations were injected into the chip. After 48 h, the cells were flushed out of the chip. Total RNA was extracted from the cells using TransZol Up solution according to the manufacturer's instructions.

The procedure was performed without RNase. Total RNA was reverse-transcribed to cDNA using TransScript® One-Step gDNA removal and cDNA Synthesis Super Mix (Paiying Quanjin Biotechnology Co., LTD.). The reaction system is shown in Table 1. The reaction conditions were as follows: The sample was subjected to denaturation for 30 s at a temperature of 94 °C, followed by 40 cycles of 5 s at a temperature of 94 °C and

30 s at a temperature of 60 °C. This process was conducted on a Real-time Thermal Cycler 5100 (Thermo Fisher Scientific) using SYBR-Green master mix. The primer sequences (*β-actin* as the internal reference gene) are shown in Table 2. The expression levels of target genes were determined using the $2^{-\Delta\Delta C_q}$ method, which calculates the relative differences.

Immunofluorescence staining

HepG2 cells were grown in 6-well plates and then incubated with different concentrations of OI for 48 h. The cultured cells were incubated in 4% paraformaldehyde for 10 min and rinsed with PBS three times. The fixed cells were blocked with 1% bovine serum albumin for 1 h.

Table 2 Primer sequences for the target genes

Gene	Primer Sequence	
	Forward primer	Reverse primer
<i>β-actin</i>	5'-TGTGACGTGGACATCCG-3'	5'-ATCTTCATTGTGCTGGGTG-3'
<i>VEGF</i>	5'-ATCTTCATTGTGCTGGGTG-3'	5'-TTCACCACTTCGTGATGATTCTG-3'
<i>MDM2</i>	1TrA1TrAAAGTCTGTGGTGCA-3	5'-TGAAGG1TITCTC1TrCCTGAAG-3'
<i>P53</i>	5'-CTTTGAGGTGCGTGT-3'	5'-CAGTGCTCGCTTAGTGC-3'
<i>Bax</i>	5'-TGAACCGGCATCTGCACAC-3'	5'-AACATGTCAGCTGCCACTCG-3'
<i>Bcl-2</i>	5'-TGAACCGGCATCTGCACAC-3'	5'-TGAACCGGCATCTGCACAC-3'
<i>Caspase-3</i>	5'-CTGGACTGTGGCATTGAGAC-3	5'-ACAAAGCGACTGGATGAACC-3'
<i>HIF-1α</i>	5'-GCTGGCCCCAGCCGCTGGAG-3'	5'-GAGTGCAGGGTCAGCACTAC-3'
<i>EGFR</i>	5'-ATCTGCCTCACTCCACCGT-3'	5'-GCAGGTACTGGGAGCCAATA-3'

Next, the cells were incubated with anti-p53 antibody at 4 °C overnight, rinsed with PBS, and incubated with FITC-conjugated secondary antibody for 20 min at room temperature. The experimental procedure for anti-VEGF antibody was the same as above.

Results

OI suppresses the migration of HepG2 cells

ImageJ software was used to process the image brightness contrast, calculate the cell migration distance of each group, and take the average of the migration number of three repeated experiments to calculate the relative migration rate of each component to the control group. The present study demonstrates that the cell migration distance of the OI-treated group displayed a statistically significant reduction as compared to the control group. The migration rates of each group were also found to be considerably lower in magnitude. The aforementioned outcomes are graphically displayed in Fig. 5, providing evidence of our findings.

OI suppresses the invasion of HepG2 cells

The chip adopted in this study used a polycarbonate membrane to simulate an artificial diaphragm to study the effect of OI on the invasion activity of liver cancer cells. The average results of three experiments were taken to calculate the relative invasion rate, and statistical analysis was performed to obtain the results of cell invasion activity under the intervention of different drugs, as shown in Table 3 and Fig. 6. Compared with the control group, the number of transmembrane cells decreased in

Table 3 Effects of different administration groups on the relative invasion rate of HepG2 cells ($\bar{x} \pm s$, $n = 3$)

Group	OD value	Relative invasion rate (%)
Control	0.427 ± 0.021	100.0 ± 3.42
Taxol	0.098 ± 0.005	22.6 ± 1.26***
OI	0.136 ± 0.005	31.92 ± 1.31***

Compared with the control group, *** $P < 0.001$

the drug site administration group, and the cell invasion ability was significantly weakened.

OI suppresses the neovascularization of HepG2 cells

Detection of cell morphology

After the perfusion and culture of HepG2 cells and HUVECs, the cell neovascularization chip was placed under an inverted fluorescence microscope for observation and photography on day 5 of culture to investigate the cell morphology, as shown in Fig. 7. The results indicated that HepG2 cells grew in clusters under 3D growth conditions coated with Matrigel. HUVECs were fusiform and formed vascular structures.

Tube formation in HUVECs

A tube formation assay was used to investigate the tube formation process of new blood vessels. The cells in the control group grew stably, and the cells were evenly distributed and filled the observation field. Compared with the control group, the HUVECs in the VEGF₁₆₅ group showed vein-like radioactive growth, and the number of

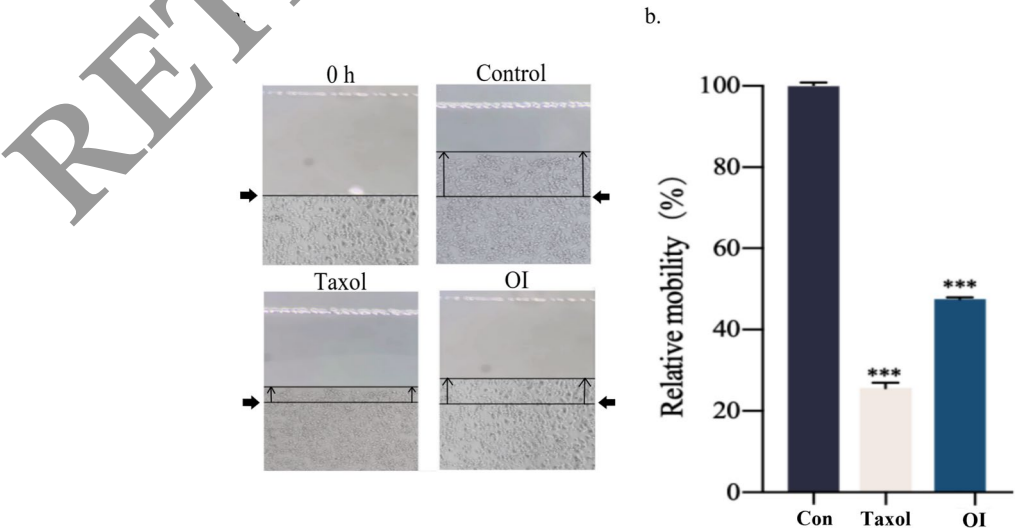


Fig. 5 OI suppresses the migration of HepG2 cells **a.** OI reduces HepG2 cell migration **b.** Effect of OI on the relative migration of HepG2 cells ($\bar{x} \pm s$, $n = 3$). Compared with the Control group, *** $P < 0.001$

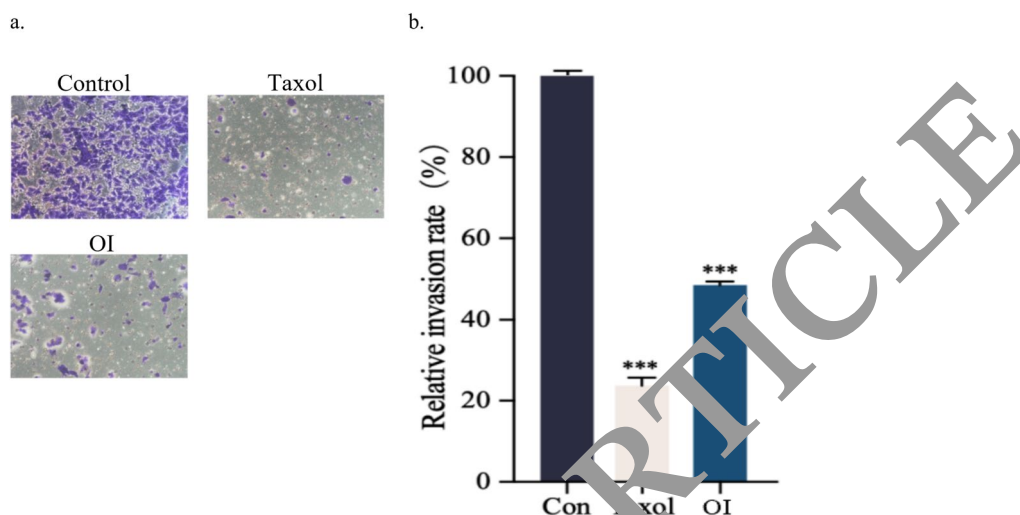


Fig. 6 Effect of OI on the invasion of HepG2 cells **a.** OI reduces HepG2 cell invasion **b.** Effect of OI on the relative invasion rate of hepatocellular carcinoma HepG2 cells ($\bar{x} \pm s$, $n=3$). Compared with the control group, *** $P<0.001$

lumen nodes and junction crossing points were significantly increased. Compared with the VEGF₁₆₅ group, the cells in the low-, medium- and high-dose OI group had smaller blood vessels, and the number of lumen nodes and the ratio of cavity area were significantly reduced. The results showed that the number of lumen nodes and the number of lumen junction crossing points in different OI groups were significantly reduced (Figs. 8 and 9).

ELISA

To evaluate whether OI influences the relative expression of VEGF and HIF-1 α , cells were exposed to OI at various concentrations (0.2, 0.5 and 0.8 mg·mL⁻¹) and then tested with ELISA. The results indicated that the relative expression of VEGF and HIF-1 α decreased gradually as the OI concentration increased (Fig. 10).

Molecular mechanism prediction

Chemical active ingredient of OI

A total of 14 potential active ingredients were screened using TCMSP, and the pharmacology database of traditional Chinese medicine systems and active ingredient information are shown in Table 4.

Construction of network

To obtain protein interaction data information, the target genes of the active ingredients were imported into the STRING database, where only the highest confidence protein interaction parameter score >0.9 was selected to construct the protein–protein interaction (PPI) network, as shown in Fig. 11a. The obtained tsv files were imported into Cytoscape software and sorted by degree from most to least. The top 10 genes were AKT1, TP53,

FOS, HIF1A, PTGS2, CASP3, MMP9, NOS3, HSP90AA1 and IL-4, which may be key anti-liver cancer target genes of OI.

A total of 257 results were obtained by GO function analysis, including 180 biological processes (BP), 23 cellular components (CC), and 54 molecular functions (MF). Order the P values from large to small, and perform visual analysis, as shown in Table 5 and Fig. 11b. The results showed that BP was mainly involved in apoptosis, transcription of the RNA polymerase II promoter, and positive regulation of interleukin IL-18 biosynthesis. MF was mainly concentrated in REDOX enzyme activity, transcription factor activity, and histone kinase activity. CC is mainly concentrated in the perinuclear region. Enrichment analysis of the KEGG pathways is shown in Table 6 and Fig. 11c. Network pharmacological screening showed that OI acted on the P53 signaling pathway, HIF-1 signaling pathway, immune signaling and other key signaling pathways through the regulation of AKT1, TP53, CASP3, MMP9, IL-4, etc., exerting anti-inflammatory, anticancer and antioxidant effects. The TP53 core target can block the cell cycle, accelerate cell senescence, promote cell apoptosis, and inhibit excessive tumor cell proliferation [27]. The HIF-1/VEGF pathway plays a key regulatory role in neovascularization of liver cancer and promotes the growth and metastasis of liver cancer tissues. Abnormal increases in VEGF, EGFR, and other angiogenic factors in tumor cells may promote tumor neovascularization and stimulate tumor growth. Therefore, in this study, the P53 and VEGF pathways were selected to explore the correlation between the mechanism of OI action on hepatoma cell apoptosis, migration, invasion, and neovascularization.

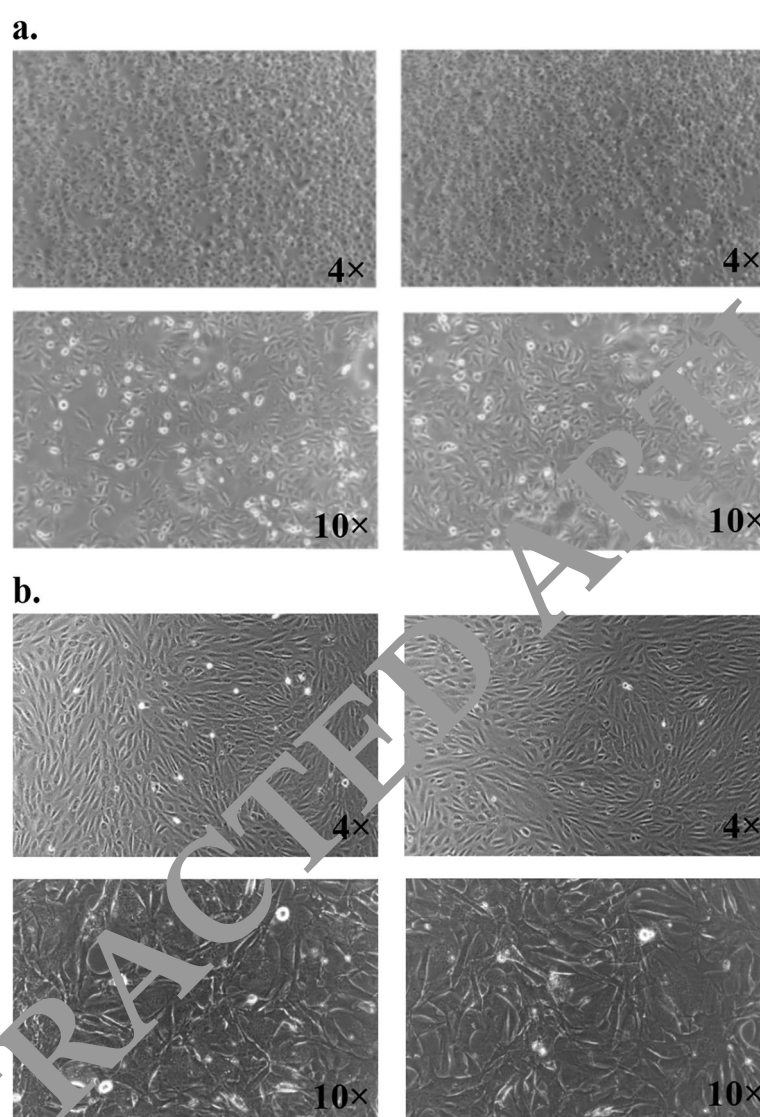


Fig. 7 Detection of cell morphology a. Morphology of HepG2 cells in the culture model b. Morphology of HUVECs in the culture model

The effects of OI at different concentrations on the expression of P53 pathway- and VEGF pathway-related genes in hepatoma HepG2 cells are shown in Fig. 12. As shown in the figure, compared with the control group, OI can significantly upregulate the expression of *Bax*, *P53* and *Caspase-3* genes and significantly downregulate the expression of *Bcl-2*, *MDM2*, *VEGF*, *HIF-1 α* and *EGFR* genes.

Immunofluorescence staining

FITC-positive expression was expressed as green fluorescent particles in the cells.

As shown in Fig. 13, OI was able to increase the number of P53-positive cells and decrease the number of

VEGF-positive cells, suggesting that the inhibitory effect of OI on HepG2 cells may be achieved by regulating P53 and VEGF.

Discussion

Cancer is one of the leading causes of death worldwide. Nonetheless, the high cost of cancer treatment is a major constraint on patients' access to quality health care in low-income countries. These populations tend to prefer traditional complementary medicines that are relatively affordable. OI is native to the Indian subcontinent at the foot of the Himalayas and extends into the ecoregions of southern China, Indo-China, and Malaysia. The plant is reported to have a wide range of biological activities

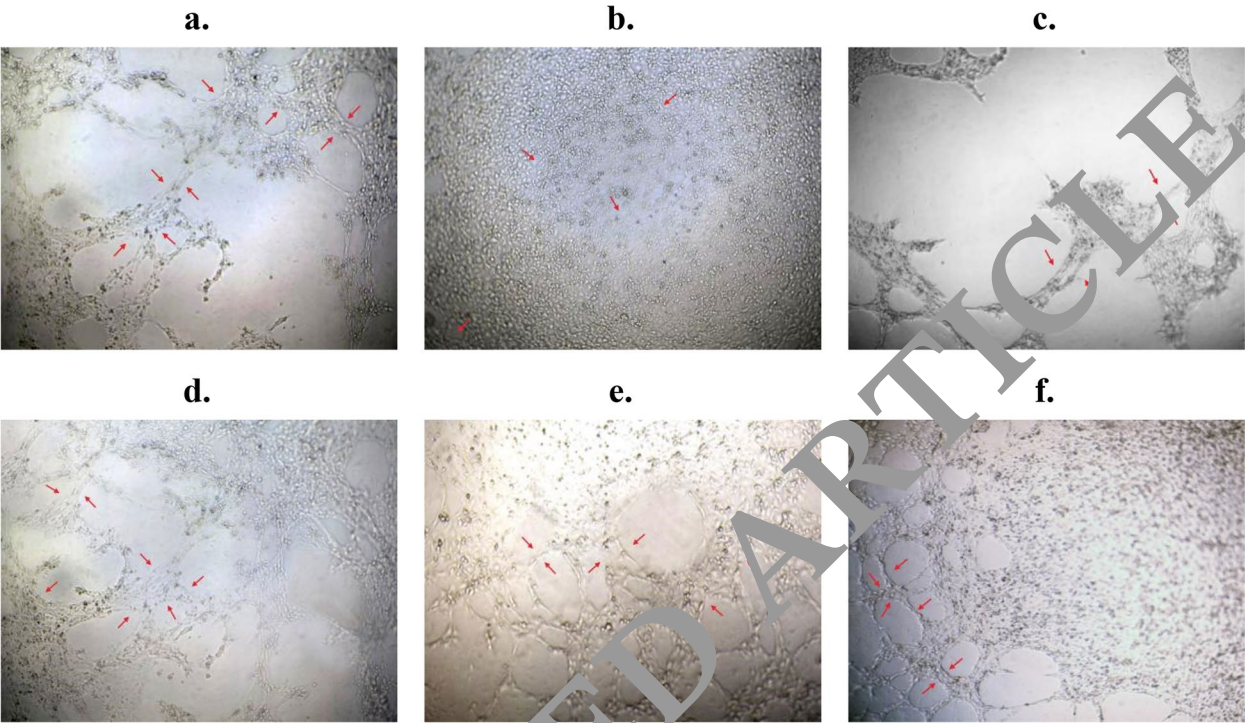


Fig. 8 Tube formation in HUVECs **a.** Control group **b.** Sorafenib group **c.** VEGF₁₆₅ group; **d.** Low dose group of OI (0.2 mg·mL⁻¹) **e.** Middle dose group of OI (0.5 mg·mL⁻¹) **f.** High dose group of OI (0.8 mg·mL⁻¹)

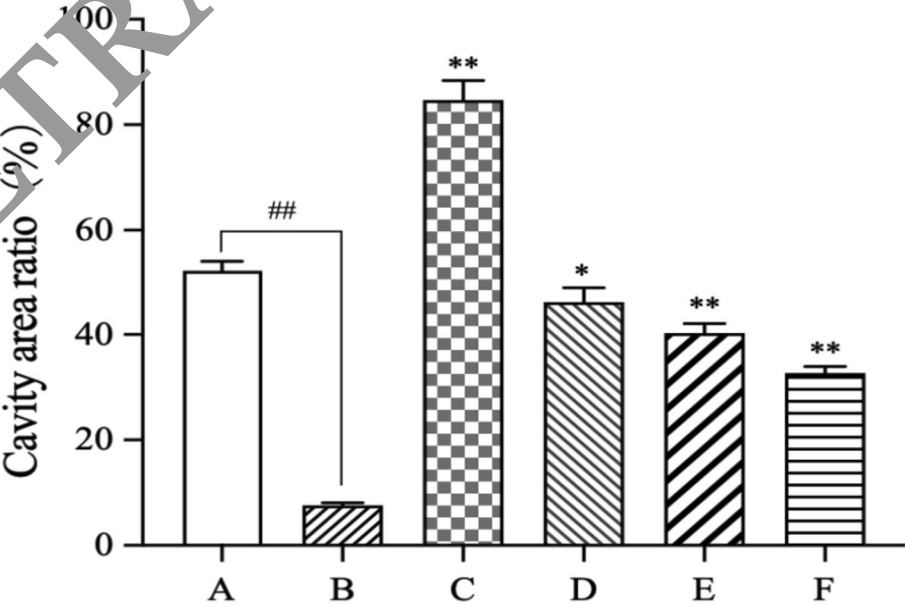


Fig. 9 Cavity area ratio compared with the control group, ^{##}*P* < 0.01. Compared with the VEGF₁₆₅ group **P* < 0.05, ***P* < 0.01 **A.** Control group **B.** Sorafenib group **C.** VEGF₁₆₅ group **D.** Low dose group of OI (0.2 mg·mL⁻¹) **E.** Middle dose group of OI (0.5 mg·mL⁻¹) **F.** High dose group of OI (0.8 mg·mL⁻¹)

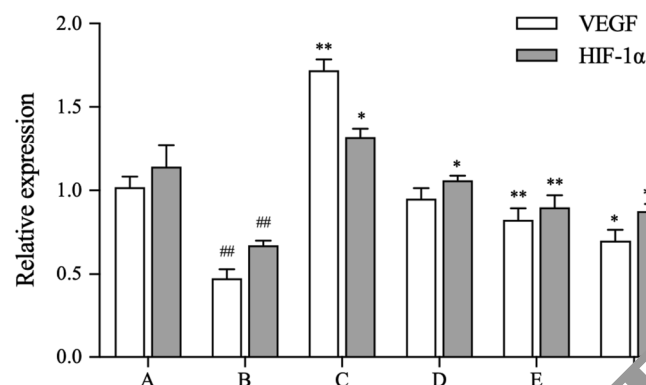


Fig. 10 Relative expression of VEGF and HIF-1α compared with the control group, ^{##}*P* < 0.01. Compared with the VEGF₁₆₅ group ^{*}*P* < 0.05, ^{**}*P* < 0.01. **A.** Control group **B.** Sorafenib group **C.** VEGF165 group **D.** Low dose group of OI (0.2 mg·mL⁻¹) **E.** Middle dose group of OI (0.5 mg·mL⁻¹) **F.** High dose group of OI (0.8 mg·mL⁻¹)

and has been used in complementary medicine to treat human diseases [28]. OI, as a traditional medicine in Asian ethnic medical systems, has been used to prevent and treat a variety of diseases, such as arthritis, rheumatism, gastric ulcers, tumors, and diabetes [12]. Interestingly, it has been speculated that the effect of OI on hepatocellular carcinoma cell proliferation may be attributed to the presence of flavonoids, including baicalin, which has previously been reported to have anticancer effects [29].

Tumors are characterized by abnormal cell growth in complex microenvironments, and traditional cell cultures are insufficient to simulate tumor cell growth in vivo. Tumor microenvironment simulation microfluidic chips have the advantages of a controllable microenvironment and high throughput. It can simulate the tumor microenvironment and build a microfluid bionic experiment

platform. In this paper, microfluidic chips were constructed to simulate the extracellular matrix environment in tissue to obtain more realistic and reliable models. Because of their high throughput, microfluidic chips can realize the function of simultaneously cultivating different cells or administering different drugs. Moreover, it is convenient to carry, has less reagent consumption, is convenient for real-time detection, and saves samples and reagents [30], which is particularly important for the field that genomics needs to face microanalysis problems. In this study, pharmacodynamic research chips were designed, namely, a cell migration chip, a cell invasion chip and a chip for RT-qPCR analysis, which provided a new technical platform for the study of tumor migration and invasion.

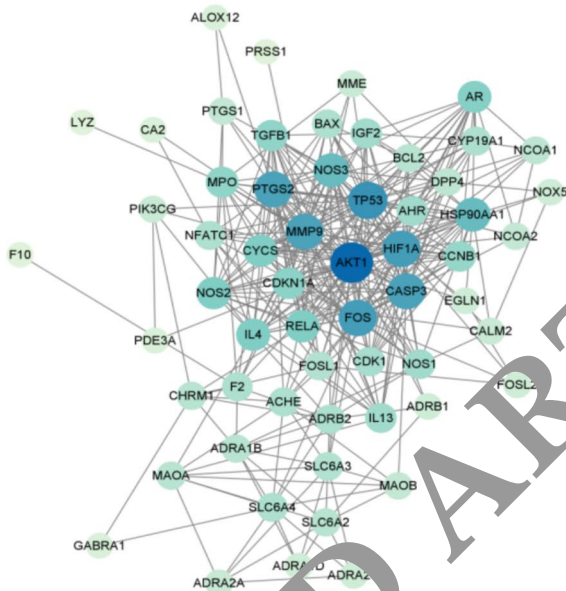
Network pharmacological screening showed that the active components of OI acted on the cancer pathway, P53 signaling pathway, HIF-1 signaling pathway, immune signaling and other key signaling pathways through the regulation of AKT1, TP53, CASP3, MMP9, IL-4, and so on, exerting anti-inflammatory, anticancer and antioxidant effects. The core target TP53 can block the cell cycle, accelerate cell senescence, promote cell apoptosis and inhibit excessive proliferation of tumor cells [27]. The HIF-1/VEGF pathway plays a key regulatory role in the formation of liver cancer neovascularization and promotes the growth and metastasis of liver cancer tissues. Abnormal increases in VEGF, EGFR and other angiogenic factors in tumor cells can promote tumor neovascularization and stimulate tumor growth. Therefore, in this study, the P53 and VEGF pathways were selected to explore the correlation between the mechanism of action of OI on hepatoma cell apoptosis, migration, invasion, and neovascularization.

HCC is characterized by high vascularization, and VEGF plays an important role in vascularization.

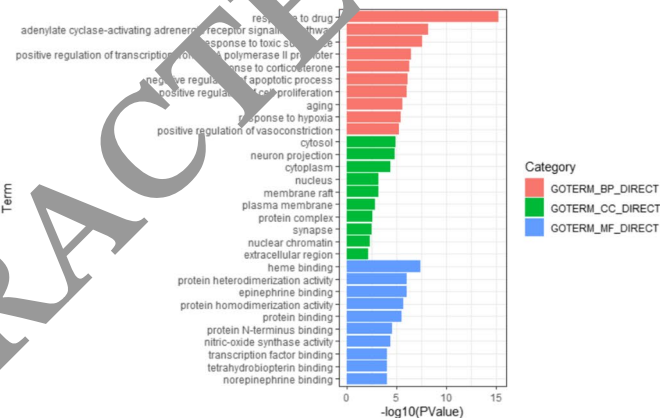
Table 4 Information on the active ingredients from OI

MOL-ID	Component name	OB(%)	DL
MOL00147	Monomodin	83.38	0.24
MOL0130	3,7,3',5'-tetramethoxy-2hydrochroxyflavone	53.26	0.42
MOL00400	6-OH-Luteolin	46.93	0.28
MOL000098	Quercetin	46.43	0.28
MOL013058	2,5-dihydroxy-6,7-dimethoxyflavone	41.52	0.28
MOL002928	Oroxin A	41.37	0.23
MOL012108	Negletein	41.16	0.23
MOL002776	Baicalin	40.12	0.75
MOL013061	Baicalein-6-glucuronide	38.72	0.76
MOL003044	Chryseriol	35.85	0.27
MOL012101	Mosloflavone	34.04	0.26
MOL002714	Baicalein	33.52	0.21
MOL001735	Dinatin	30.97	0.27
MOL000173	Wogonin	30.68	0.23

a.



b.



c.

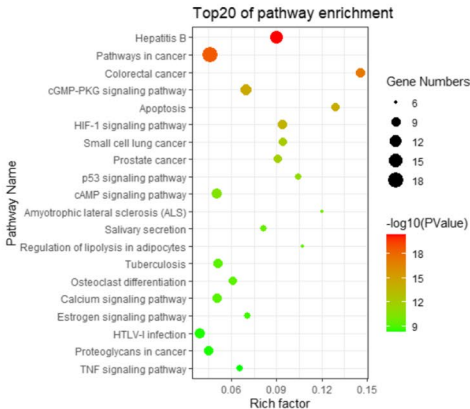


Fig. 11 Construction of network **a**. PPI diagram: The bait proteins are marked by large blue nodes. Connecting lines show the number of interactions (direct or shared prey proteins) between bait genes. **b** GO function analysis **c**. KEGG function analysis

Table 5 GO analysis of core targets

Category	Gene function	Count	P value
BP	positive regulation of apoptotic process	7	8.21×10^{-7}
BP	platelet activation	5	9.58×10^{-6}
BP	positive regulation of protein phosphorylation	5	1.4×10^{-4}
BP	negative regulation of gene expression	5	1.64×10^{-4}
BP	positive regulation of MAPK cascade	4	3.33×10^{-4}
BP	negative regulation of extrinsic apoptotic signaling pathway	3	8.66×10^{-4}
BP	negative regulation of cell growth	4	1.01×10^{-3}
BP	positive regulation of mononuclear cell migration	2	1.47×10^{-3}
BP	positive regulation of angiogenesis	3	6.74×10^{-3}
MF	iron ion binding	5	2.41×10^{-4}
MF	RNA polymerase II transcription factor binding	3	1.29×10^{-3}
MF	cyclin binding	2	7.09×10^{-3}
CC	protein complex	7	2.72×10^{-4}
CC	extracellular region	13	7.33×10^{-4}

Vascular growth in developmental, tissue repair, or disease states involves the germination, migration, and proliferation of endothelial cells, which are regulated by a variety of factors, among which VEGF plays a crucial role [31]. As an important promoter in angiogenesis, VEGF can promote endothelial cell mitosis, increase vascular permeability, promote capillary fusion, and induce angiogenesis. Hypoxia-inducing factor HIF-1 α can induce

endothelial cells and cancer cells to secrete angiogenesis-related factors, thus leading to changes in the tumor microenvironment and inducing tumor growth. HIF-1 α is highly expressed in hemangioblastoma, glioblastoma multiforme, colorectal adenocarcinoma, and subtypes of breast, prostate, and lung cancer [32].

In this study, microfluidic chips were used to culture cells, and then subsequent target gene detection was conducted. The RT-qPCR results showed that OI can inhibit the migration, invasion and neovascularization of HepG2 cells, and its regulatory mechanism may be related to the regulation of the P53 and HIF-1/VEGF pathways, as shown in Fig. 14 by FigDraw (<https://www.figdraw.com>).

Bax serves as a catalyst for apoptosis, significantly impacting the response of cancer cells to chemotherapeutic agents, and thus has a direct correlation with the clinical prognosis of cancer patients [33]. The activation of P53 plays a crucial role in regulating a wide range of cellular functions, including metabolism, apoptosis and nonapoptotic cell death, as well as migration and invasion, ultimately resulting in a tumor-suppressive effect [34]. P53 has emerged as a significant antitumor factor that plays a crucial role in cancer prevention and treatment. Activation of p53 regulates the expression of multiple genes associated with cancer initiation and progression. Indeed, accumulating evidence suggests that p53 plays a vital role in inhibiting cancer development and progression. Under homeostatic conditions, the stability and function of p53 are tightly regulated by MDM2, which targets p53 for degradation and directly inhibits p53 activity by binding to its transcriptional activation domain. MDM2, together with its binding partner MDMX, maintains p53 activity by limiting the

Table 6 Pathway enrichment analysis of primary targets

NO	KEGG signaling pathway	Count	P value
1	Hepatitis B	13	8.33×10^{-8}
2	Pathways in cancer	18	4.15×10^{-8}
3	Colorectal cancer	8	7.40×10^{-7}
4	cGMP-PKG signaling pathway	11	7.41×10^{-7}
5	Apoptosis	8	9.57×10^{-7}
6	HIF-1 signaling pathway	9	3.80×10^{-6}
7	Small cell lung cancer	8	1.12×10^{-5}
8	Prostate cancer	8	2.21×10^{-5}
9	p53 signaling pathway	7	4.57×10^{-5}
10	cAMP signaling pathway	10	7.88×10^{-5}
11	Amyotrophic lateral sclerosis	6	8.25×10^{-5}
12	Salivary secretion	7	9.76×10^{-5}
13	Regulation of lipolysis in adipocytes	6	1.79×10^{-4}
14	Tuberculosis	9	2.79×10^{-4}
15	Osteoclast differentiation	8	2.81×10^{-4}
16	Calcium signaling pathway	9	4.01×10^{-4}
17	Estrogen signaling pathway	7	4.23×10^{-4}
18	HTLV-I infection	10	5.57×10^{-4}
19	Proteoglycans in cancer	9	6.07×10^{-4}
20	TNF signaling pathway	7	7.01×10^{-4}

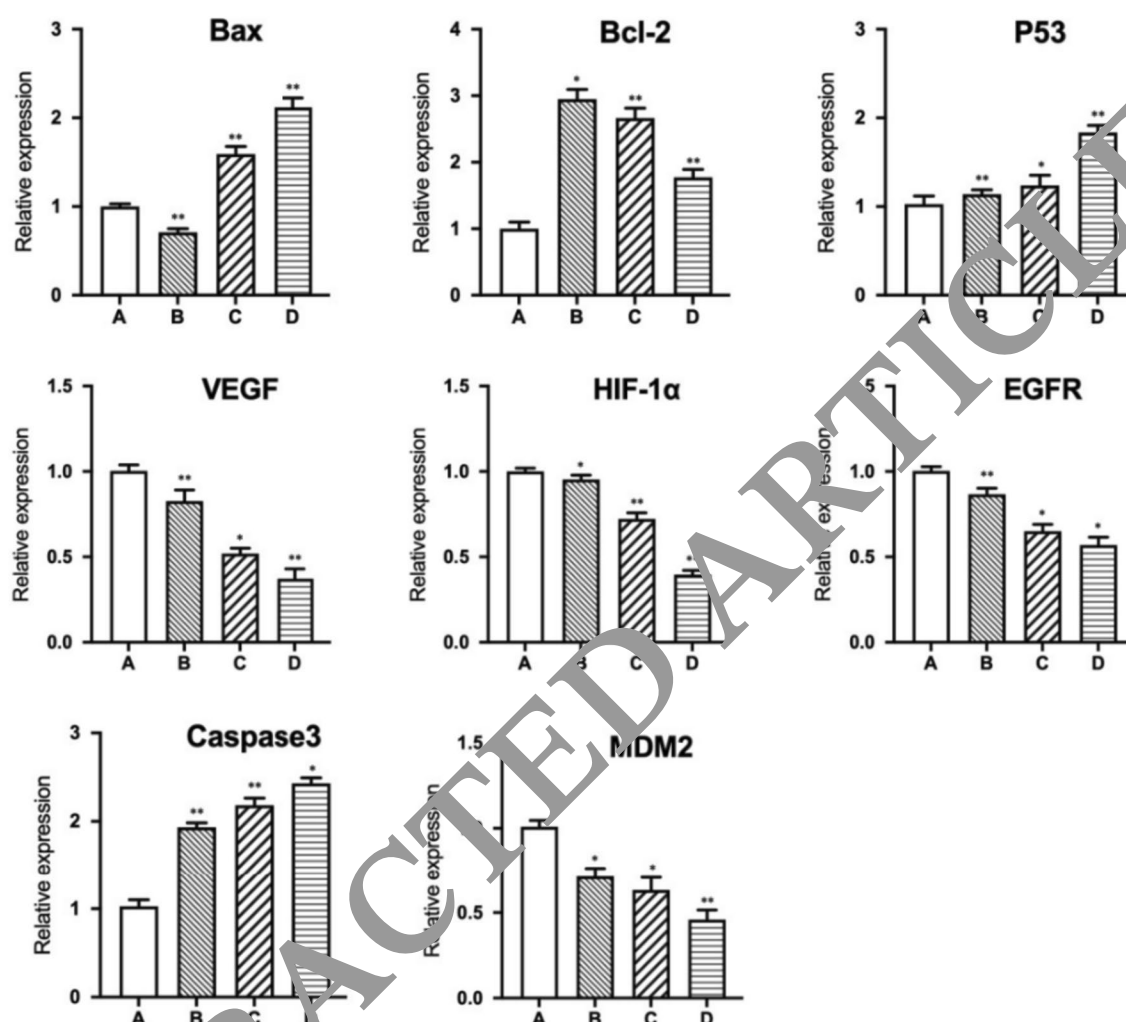


Fig. 12 Effect of OI on relative gene expression in HepG2 cells **A.** Control group; **B.** Low-dose OI group; **C.** Medium-dose OI group; **D.** High-dose OI group. Compared with the control group, * $P<0.05$, ** $P<0.01$

transcription factor response and establishing an effective feedback loop [35]. *Caspase 3* is a downstream effector cysteine protease that plays a crucial role in the apoptotic pathway of hepatocytes, making it a major executor of programmed cell death. The activation of caspase 3 is fundamental to the apoptotic process and is involved in the development and progression of hepatocellular carcinoma, highlighting its pivotal role in the apoptotic mechanism [36]. *Bcl-2* is renowned for its ability to impede apoptosis, a critical mechanism that regulates programmed cell death [37]. Research studies have conclusively demonstrated that angiogenesis plays a crucial role in the progression of hepatocellular carcinoma. VEGF, a critical therapeutic target for cancer, is an indispensable angiogenic factor in hepatocellular carcinoma. HIF-1α, a pivotal player in hypoxia-related signaling pathways, notably contributes to angiogenesis

in hepatocellular carcinoma. Hypoxia-induced hepatocellular carcinoma cells exhibit heightened levels of HIF-1α. HIF-1α possesses the potential to stimulate angiogenesis by inducing VEGF expression [38]. *EGFR* serves as a central hub for the convergence of several extracellular growth and survival signals, thereby orchestrating cellular responses. EGFR plays a crucial role in a wide variety of aspects of cellular growth and survival, such as proliferation, survival, and migration, and its overexpression has been linked to highly aggressive and invasive tumors but is also associated with reduced survival rates [39]. OI inhibits the migration and invasion of HepG2 cells via the P53 and VEGF pathways while significantly upregulating the expression of the *Bax*, *P53* and *Caspase-3* genes and down-regulating the expression of the *Bcl-2*, *MDM2*, *VEGF*, *HIF-1α* and *EGFR* genes.

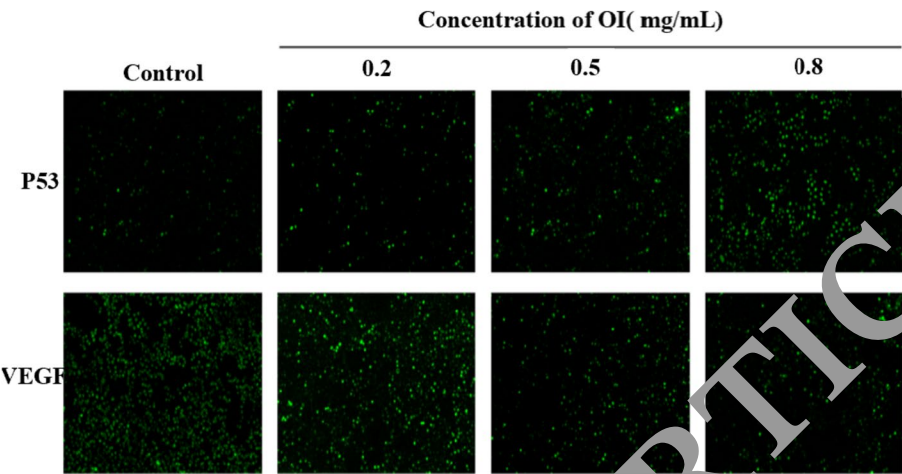


Fig. 13 Immunofluorescence staining The immunofluorescence staining method of secondary antibody indirect staining labeled by FITC was adopted, which can stimulate green fluorescence at 488 nm wavelength with high sensitivity and flexible staining. OI may inhibit the progression of liver tumors by regulating P53 and VEGF expression

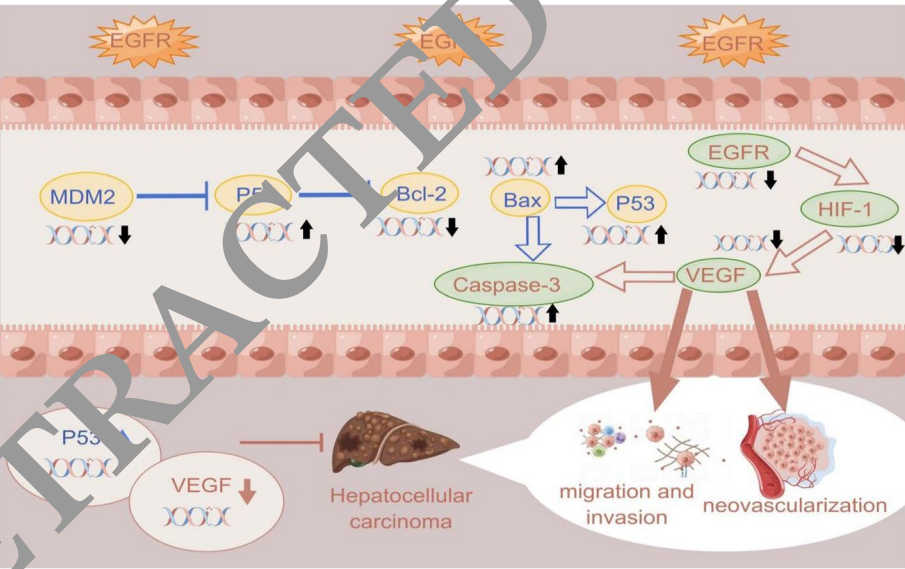


Fig. 14 Mechanism of OI in the treatment of liver cancer. OI inhibits the migration, invasion and neovascularization of HepG2 cells by activating the P53 pathway and blocking the VEGF pathway

The present study highlights the potential use of OI against HCC. The established strategy in this study also lays a great foundation for the future investigation of OI, which provides further development and application of TCM for the therapy of HCC.

Abbreviations

- BP Biological processes
- CC Cellular components
- ELISA Enzyme-linked immunosorbent assay
- FITC Fluorescein isothiocyanate
- HCC Hepatocellular carcinoma

- HepG2 Hepatocellular carcinomas
- HUVEC Human umbilical vein endothelial cells
- MF Molecular functions
- OI Oroxylum indicum
- PPI Protein–protein interaction
- QOL Quality of life
- RT–PCR Reverse transcription–polymerase chain reaction
- TAI Tumor neovascularization inhibitors
- TCM Traditional Chinese medicine
- TIC Total ion currents

Acknowledgements

We wish to thank all the staff members of Liaoning Multidimensional Analysis of Traditional Chinese Medicine Technical Innovation Center, Dalian, China.

Authors' contributions

Funding acquisition, Xiansheng Meng; conceptualization, Xi Luo and Yi Zheng; data curation, Miao Zhao and Qiang Zhang; investigation, Miao Zhao and Xi Luo; methodology, Xiansheng Meng and Yongrui Bao; resources, Xiansheng Meng; writing-original draft, Xi Luo and Sicong Liu; writing-review and editing, Shuai Wang and Tianjiao Li. All authors reviewed the manuscript.

Funding

This study was supported by grant 81874342 from the National Natural Science Foundation of China.

Availability of data and materials

The datasets are included in this article. The algorithms used to process the data are available from the corresponding author upon reasonable request. Please send the permission document to mxsvvv@163.com.

Declarations

Ethics approval and consent to participate

All experimental studies on plants complied with relevant institutional, national, and international guidelines and legislation.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 12 May 2023 Accepted: 13 October 2023

Published online: 07 November 2023

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