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# Isorhamnetin Inhibits the Proliferation and Induces Apoptosis of Hepatocellular Carcinoma by Targeting the GSK3-β/PI3K/AKT Pathway

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## Abstract

The natural compound possesses quality therapeutic effects and few side effects, development of new uses for marketed drugs, is an effective way to discover new antitumor compounds. Here, we have investigated the mechanism of Isorhamnetin (ISO) in the prevention and treatment of Hepatocellular Carcinoma (HCC). *In-vitro* cell models were used to measure the regulation effects of ISO on HCC, CCK-8 proliferation assay, wound healing assay and Transwell invasive assay were used to choose the of candidate cell lines. Flow cytometric analysis of apoptosis, and Western blot were performed to elucidate the mechanism of ISO. The targets on HCC was predicted using network pharmacology and molecular docking analyses. The putative pathways were further verified *in vivo* assays. The *vitro* experiments were demonstrated that ISO potently suppressed the proliferation, colony formations, wound healing well as invasive process in HCC. Network and biological analyses predicted that one of the core mechanisms about ISO on HCC was via regulation of cancer cell apoptosis, western blotting validated ISO inhibited HCC *via* GSK-β/PI3K/AKT pathway. Also, in mouse hepatocellular carcinoma xenograft model, ISO inhibited tumor growth with few side effects. These findings suggest that ISO is the natural anti-cancer compounds to inhibitor HCC effectively through the GSK-β/PI3K/AKT signaling pathway. Natural anti-cancer therapy maybe a promising strategy for the prevention and treatment of HCC.

**Keywords: Isorhamnetin; Network pharmacology; Molecule docking; Hepatocellular carcinoma; GSK-β/PI3K/AKT signaling pathway**

## Introduction

Hepatocellular Carcinoma (HCC) is one of the most common invasive human malignancies, a primary malignancy of the liver, and it is the third cause of cancer-related deaths worldwide, with more than 800,000 mortalities per year, making it the third cause of cancer-related deaths worldwide [1]. In the early stages, the systemic chemotherapy is still the most effective treatment [2]. Although remarkable endeavor has been made for seeking therapeutic strategies and combating liver cancer, the great majority of synthesized chemotherapeutic agents have serious adverse effects. Metastasis is still a major clinical challenge that accounts for the primary cause of cancer-related mortalities, particularly for Hepatocellular carcinoma. Herbal medicines, which are made from purely natural constituents, always have fewer side effects for the therapy of cancer [3]. Many plant-derived drugs with excellent properties have been accepted as potential alternatives for the treatment of hepatocellular carcinoma [4]. Therefore, novel potential biomarkers and treating strategies for HCC therapy need to be urgently identified.

Natural products derived from plants have become an important source for drug development because their secondary metabolites are diverse and complex chemical components with good biological activity. Natural products were widespread attention in the field of anti-cancer treatment owing to their low toxicity, multiple targets, and curative effects [5]. and the effects of ISO on HCC

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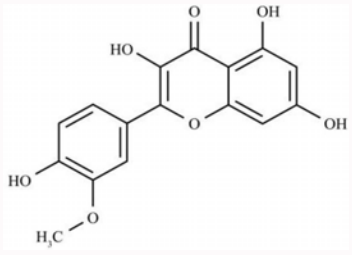


**Figure 1:** Molecular structure of Isorhamnetin.

have not been widely studied. Isorhamnetin, a flavonoid that can be isolated from the plants of ginkgo leaves. Chemical formula is found in Figure 1. This compound has numerous pharmacological effects, and exhibited the highest cytotoxic activity against most of cancer cell lines likes gallbladder cancer, breast cancer, ovarian cancer, and colon cancer [6-8]. In the previous ten years, a variety of Omic technologies for the high-throughput screening and identification of targets involved in natural plant medicine. One of the principal applications of system biology has been to better understand the complex mechanisms of action of natural herb medicine is using network pharmacology. For example, Trabedersen have reached preclinical and clinical trials for cancer and autoimmune disorders therapy though network pharmacology in the last few years [9]. Natural products source offers abundant antioxidants for disease treatment [10]. Network pharmacology combined metabolomics to study the pharmacological mechanism of Shengqi Fuzheng injection regulation of mitochondrial dysfunction in CRF [11]. The systems pharmacology method makes pathway-based drug discovery quick and effective [12]. This study elucidated how ISO stimulates apoptosis, and inhibits metastasis of liver cancer cells *in vitro* and *in vivo* in a dose-dependent approach.

## Materials and Methods

### Reagents

The Isorhamnetin (ISO) (purity >98.64%) was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). 5-Fluorouracil (5-FU) was purchased from Shanghai Aladdin Chemical Reagent Co., Ltd., (Shanghai, China).

### Cell line and Cell culture

HepG-2, MHCC97-H and LO2 were presented from institute of tropical medicine, (Guangzhou University of Chinese Medicine, Guangzhou, China), and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) at 37°C in a humidified atmosphere of 5% $CO_2$. Cells were passaged or expanded by trypsinization when they grew to 80% confluence.

### Cell proliferation assay

For CCK-8 assays, cells were seeded at a concentration of 5,000 cells per well in 100 μL DMEM medium with 10% FBS into 96-well plates, and allowed to adhere for 24 h, then cells were treated with different concentrations of Isorhamnetin for 72 h. Cell viabilities were measured using Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) by following the manufacturer's instruction. Afterwards, the optical density OD value was measured at 450 nm using a microplate reader.

### Wound healing assay and Transwell invasive assay

In regard to wound healing assays, $4 \times 10^5$ cells were seeded onto 6-well plates for over 90% confluency and scratched with a 1-ml pipette tip. Next, cells were exposed to the indicated treatment, washed thrice with PBS, and recorded at 0, 24, and 48 h after initiation of the wound healing. The healing distance and healing area were analyzed by Image J software (National Institute of Mental Health, Bethesda, Maryland, USA). For Transwell invasive assays, 8-um Transwell chambers (Corning, New York, USA) were coated with Matrigel (Corning, New York, USA) and placed into 24-well plates before experiment. Subsequently, the upper chambers were added with cells at a density of $1.5 \times 10^5$ following the indicated treatment, whereas the lower chamber was filled with complete DMEM medium containing 10% FBS for 24 h. Finally, the upper cells were erased with a cotton swab, while the invaded cells into the bottom of chamber were fixed with 4% formaldehyde, dyed with 0.5% hematoxylin solution for 30 min, and photographed for examination.

### Colony formation assay

Cells were seeded into six-well plates at a density of 5,000 cells/well and treated with ISO at different concentrations. After 24 h, the medium was replaced with fresh complete DMEM. then cultivated with fresh complete culture medium for additional 7 days. Cell colonies were fixed in 4% paraformaldehyde for 20 min, and stained with 0.01% (w/v) crystal violet for 10 min, following by thoroughly wash with $H_2O$ and air dried, and then counted for examination.

### Flow cytometry analysis

For the drug efflux assay, cells were incubated with ISO for 24 h. For the apoptosis analysis, the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, United States) was implemented to dyed with the cells. For cell cycle analysis, cells were fixed in ice-cold 70% ethanol for 24 h. Then cells were washed with phosphate-buffered saline, stained with propidium iodide (BD Biosciences). All flow cytometry analyses were performed with BD Accuri C5 or LSRFortessa and analyzed with FlowJo software.

### Western blot analysis

RIPA Lysis buffer containing 1 mM aprotinin, 1 mM pepstatin, 1 mM NaF, 1 mM $Na_4P_2O_7$, and 1 mM $Na_3VO_4$ was implemented to dissolve the C2C12 myotubes in the different treatments. The enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China) was used to measure the protein concentration in the lysate. The quantitative protein samples (20 μg) were subjected to SDS-PAGE on a 12% polyacrylamide gel and then moved to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated with the corresponding primary antibodies including AKT and p-AKT, PI3K and p-PI3K, GSK3β and p-GSK3β (Cell Signaling Technology, Beverly, MA, USA); cleaved-caspase9, caspase9, cleaved-caspase3, caspase3, CDK2, NOS3, HSP90, bcl-2, bax, GAPDH (Abcam, USA) were used at 4°C overnight. At room temperature, the membrane was incubated with a secondary antibody (Abcam, USA) for 2 h and then washed three times with Tris-buffered saline and 0.05% Tween-20. Subsequently, using an Immobilon™ Western Chemiluminescent HRP Substrate kit the proteins were visualized (Millipore, Billerica, MA, USA). Chemiluminescent Imaging and Analysis Systems (Tanon, Shanghai, China) were used to detect the immune response signal.

### Hepatocellular carcinoma xenotransplantation model

All the animal experiments were approved by the Animal Ethics Committee at Guangzhou University of Chinese Medicine. Four-week-old BALB/c nude mice were obtained from Guangdong Medical Laboratory Animal Center. The nude mice were housed

in the specified-pathogen-free animal laboratory. After a 1-week acclimation period, MHCC-97H cells at a density of $2 \times 10^6$/ml were mixed with Matrigel (Corning, New York, USA), and implanted into the right flanks of the mice. The mice were examined every day. When the tumors reached a diameter of 0.1 $mm^3$, the nontumor mice were excluded and the remain were randomly assigned into four groups (n=5), that Intraperitoneal administration was performed every two days with vehicle (2.5% EtOH, 2.5% Cremophor EL in saline solution), Isorhamnetin lower dosage (5 mg/kg), Isorhamnetin high dosage (20 mg/kg), 5-FU (50 mg/kg) [13]. Tumor volume was measured each two days after grouping. Finally, the mice were euthanized after the $15^{th}$ days treatment, and their tumor were removed and compared.

### Hematoxylin & eosin staining and immunohistochemistry

The tumor samples were embedded in paraffin and then mounted on poly-L-lysine-coated glass slides for immunohistochemistry analysis. Then the slices were treated with xylene and different concentrations of ethanol gradually, finally immersed in distilled water. H&E staining is used to identify tissue lesions. For immunohistochemistry analysis, the sections were firstly treated with 0.025% Triton X-100, After blocking with a TBS solution containing 10% normal serum and 1% BSA, the sections were inoculated with the indicated primary antibody ki67 (Abcam, Cambridge, USA) at 4°C. DAB detection system (Dako A/S, Glostrup, Denmark) was applied as chromogenic agents according to the manufacturer's instructions. Finally, sections were counterstained using Mayer's hematoxylin, before examination.

### Screening of active targets of isorhamnetin

We used the Traditional Chinese Medicine Systems Pharmacology Database Analysis Platform (TCMSP http://lsp.nwu.edu.cn/index.php), Bioinformatics Analysis Tool for Molecular Mechanism of Traditional Chinese Medicine (BATMAN-TCM http://bionet.ncpsb.org/batman-tcm/) and PubChem (https://pubchem.ncbi.nlm.nih.gov/) to identify the active targets of Isorhamnetin. Uniport (https://www.uniprot.org/) was searched to obtain the standard target names.

### Screening of hepatocellular carcinoma-related targets

We used "Hepatocellular carcinoma" as keywords in GeneCards (https://www.genecards.org/), Therapeutic Target Database (TTD, http://bidd.nus.edu.sg/group/cjttd/), and Online Mendelian Inheritance in Man (OMIM, https://www.omim.org/) to search for Liver cancer-related targets. These were then imported into an EXCEL table for standardization for subsequent statistical analysis (Sheet 1).

### Identification of candidate genes on component-target network

To identify common active targets, we mapped the Isorhamnetin and the disease targets by using Bioinformatics & Evolutionary Genomics (http://bioinformatics.psb.ugent.be/webtools/Venn/) and constructed a Venn diagram to show putative targets of Isorhamnetin for liver cancer. We then obtained the PPI network using the STRING database (https://string-db.org/) to reveal the mechanism by which components act against Liver cancer. The following settings were used: Text-mining, Experiments, and Databases. The minimum required interaction score was 0.7, which indicated high confidence. Finally, we disabled the structure previews inside network bubbles and we hid disconnected nodes in the network. Cytoscape software were used to construct the PPI subnetwork, which showed the significant target genes. To explain the relationship between the active compounds and its target genes, this study constructed a visual "component-target" network in Cytoscape 3.2.1. In this network, compounds and targets are represented by nodes, and the interaction between two nodes is represented by edges.

### Gene ontology and Kyoto encyclopedia of genes and genomes pathway enrichment

To analyze the biological and signal pathway conduction processes in the actions of compounds against Hepatocellular carcinoma, and to clarify the mechanism of drug action, the targets in the network were imported into the DAVID database (https://david.ncifcrf.gov/) for Gene Ontology (GO) analysis (including Biological Processes [BP], Molecular Functions [MF], and Cellular Components [CC]) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. "ggplot2" packages from R 3.5.3 software was used to draw related charts. Finally, the relationship between target genes and pathways was presented as a target-pathway network diagram in Cytoscape.

### Molecular docking

The crystal structures of the hub targets were downloaded from the RCSB PDB database (PDB, http://www.pdb.org/), and the structures of the active ingredients were obtained from the TCMSP database. AutoDock 4.2 was used to take away the water molecules, isolate proteins and reserve them as receptors. the targets and the active ingredients were processed docked through the Discovery Studio software.

### Statistical analysis

All experiments were repeated at least three times. All data are presented as mean ± standard error and were analyzed by one-way ANOVA from GraphPad Prism v6.0 software (GraphPad Software, Inc., San Diego, CA, USA). The differences were considered significant at $p<0.05$, extremely significant at $p<0.01$.

## Results

### Isorhamnetin anti-proliferation effects in hepatocellular carcinoma

we investigate whether ISO could affect the growth of multiple hepatocellular carcinoma cell lines including two two-cancer phenotypes MHCC97-H and HepG-2, as well as a non-malignant liver cell LO-2. As shown in Figure 2A, 2B, ISO dose- and time-dependently suppressed the proliferation of liver cancer cell, and Inhibitory Concentration (IC50) of ISO at 48 h for MHCC97-H and HepG-2 cells were 14.179 and 12.204 μg/mL, respectively. The inhibitory effect of ISO on hepatocellular carcinoma cells was also confirmed by a colony formation assay. There was a reduced number of colonies that formed in the ISO group compared with vehicle control (Figure 2C). At a concentration of 0 μM to 30 μM, ISO expressed the colony-forming inhibiting capability on MHCC97-H cells in dosage dependent manner. However, the effect on HepG-2 cells is not obvious as MHCC97-H cells.

### Isorhamnetin attenuates migrative and invasive potential of HCCs

Another key point in of cancer cells is the ability of tumor cells to invade and metastasize. In this part, we investigated the *in-vitro* influence of ISO on the infiltration potential of both MHCC97-H cells and HepG-2 cells. As indicated in Figure 3A, in MHCC97-H cells, the gap widths and areas of the untreated group were narrowed more rapidly compared to those of the ISO group from 0 h to 48 h, implying that ISO inhibited the migrative ability of both indicated

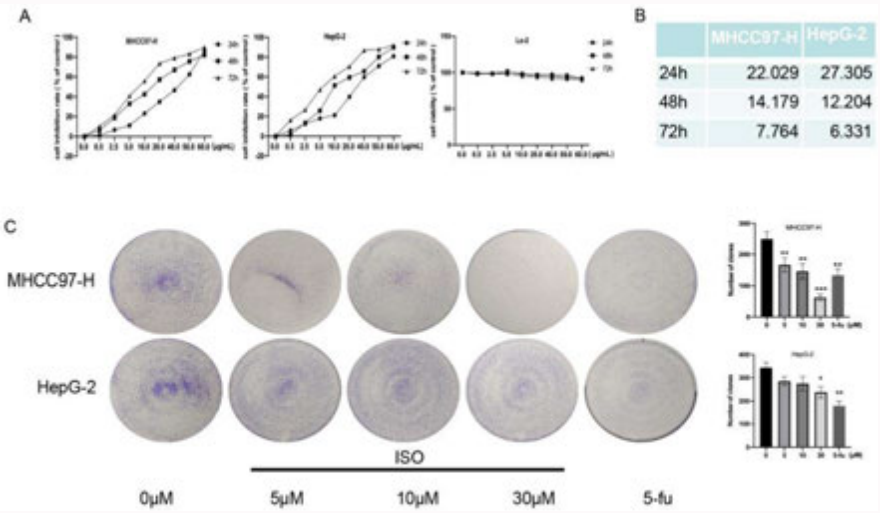


**Figure 2:** ISO exerts anti-proliferation effects in liver cancer cells. (A) Exponentially growing cells of MHCC97-H, HepG-2 and Lo-2 were treated with ISO at the indicated concentrations (0–60 μg/mL) for 24, 48, and 72 h by CCK8 assays; (B) The IC50s of ISO for MHCC97-H, HepG-2; (C) Colony formation assay for evaluating the inhibitory effects of ISO with the different concentrations (0–30 μg/mL) on MHCC97-H, HepG-2 cells. All values represent the mean ± SD (*p<0.05, **p<0.01, ***p<0.001, *vs.* control, n=3).

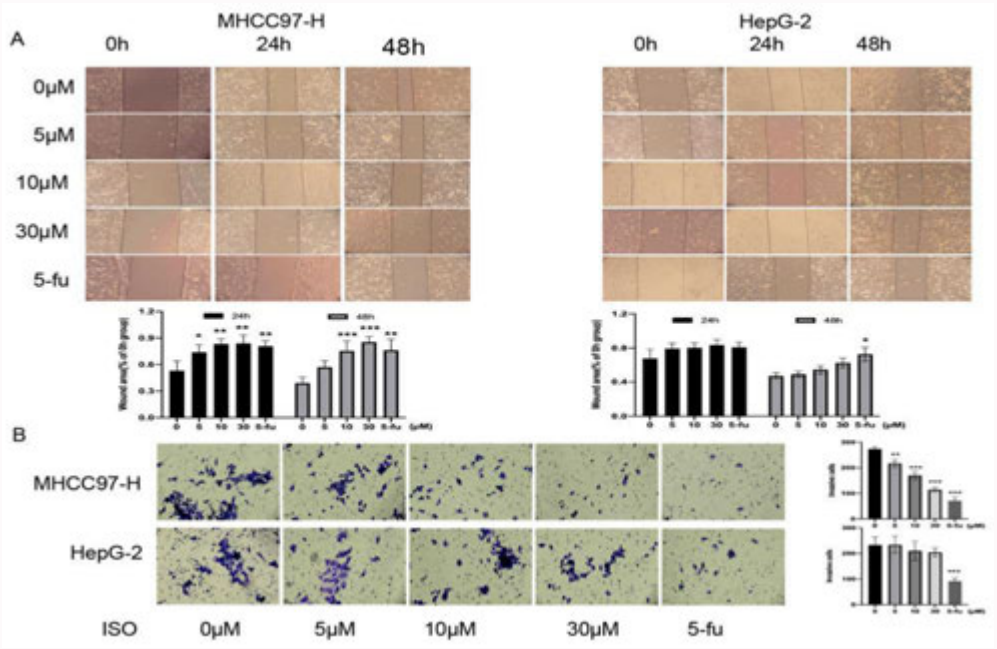


**Figure 3:** ISO suppresses migration and invasion in metastatic cells. (A) Representative phase-contrast images for wound healing assays. Left: MHCC97-H cells, right: HepG-2 cells. Quantification of migration healing areas were shown under each chart. (B) Representative images (left) and quantification (right) of decreased cell number in Transwell chambers with ISO at different concentrations (0–30 μg/mL). All values represent the mean ± SD (*p<0.05, **p<0.01, ***p<0.001, *vs.* control, n=3).

cells at a dose- and time-dependent manner. For HepG-2 cells, only ISO with the concentration at 30 μM showed a significant ability to inhibit migration within 48 h. Furthermore, chamber invasive assay showed that the invasive cell number after admission Isorhamnetin. In MHCC97-H cells, the number of invasive cells were significantly reduced following Isorhamnetin treatment at 0 μM to 30 μM, but had no effect on HepG-2 cells. Demonstrating that ISO administration weakened the invasive potential of hepatocellular carcinoma in MHCC97-H (Figure 2B). So, we chose MHCC97-H cells as the deeply research object.

## Isorhamnetin obvious anti-apoptotic in HCCs and arrest cell in G0/G1 phase

Following a 48-h administration with ISO, FACS analysis was used to determine cell cycle and apoptosis on MHCC97-H cells. As shown in Figure 4A, the proportion of G1 phase cells in the control group was lower than that in the ISO group. The drug increased G0/G1 arrest from 44.02% to 51.80%, 58.01% and 67.49% with ISO concentration on 0 μM to 30 μM, indicating that ISO can induce the cell cycle process of HCCs. Annexin V/PI staining was applied to detect the apoptotic events in HCCs. As labeled in Figure 4B,

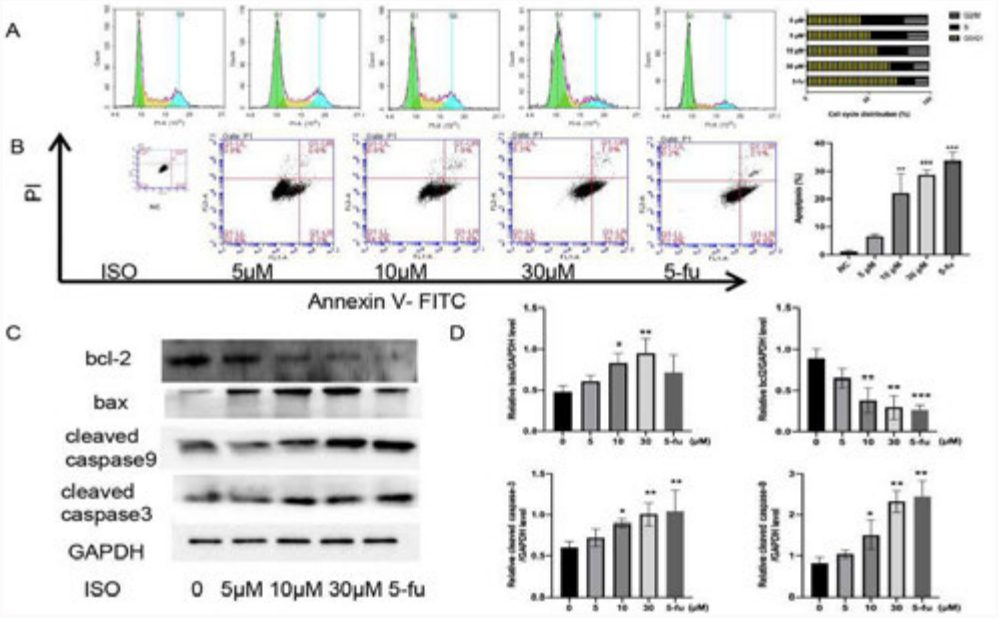


**Figure 4:** ISO exerts apoptosis-promoting and arrest cell cycle effects in hepatocellular carcinoma. (A) Cell cycle analysis of ISO (0–30 μg/ml) treated MHCC97-H cells was performed by flow cytometry. (B) The apoptotic populations after treatment ISO (0–30 μg/ml) in MHCC97-H cells. (C) Western blotting analysis of Bax, bcl-2, caspase 3 and caspase 9 as well as cleaved caspase 3, cleaved caspase 9 after the indicated ISO treatment. (D) quantification of each protein after admission different dosage ISO. All values represent the mean ± SD (*p<0.05, **p<0.01, ***p<0.001, *vs.* control, n=3).

compared with the untreated cells, a 48 h administration with 5, 10, 30 μM doses of ISO significantly increased number of apoptotic cells, the percentage of early and late apoptotic events reached about 13.7, 26.7 and 37%, respectively. Moreover, we observed that the expression of Bcl-2 was downregulated by ISO treatment in a dose-dependent manner, the expression of Bax, cleaved caspase 3 and cleaved caspase 9 was significantly increased (Figure 4C, 4D). Together, these findings confirmed that ISO promoted cell apoptosis in MHCC97-H cells.

## Construction of an ingredient-target-hepatocellular carcinoma network of isorhamnetin

To thoroughly reveal the anti-cancer mechanism of ISO, A total of 38 potential human targets were identified, 16,833 hepatocellular carcinoma-related target genes were obtained from the three disease databases. Then, a Venn diagram was constructed to match potential compound target genes with disease-related genes by Bioinformatics & Evolutionary Genomics platform (Figure 5A), and all associated genes are shown on the right side of Figure 4A. To identify the functions and mechanisms of ISO, we imported the 38 targets into the STRING database to generate a PPI network, (settings: Species Homo sapiens, confidence level >0.7, STRING database confidence level high >0.7; medium >0.4; and low >0.15). Next conducted GO and KEGG pathway-enrichment analysis based on the background of all human genes. For GO-term analysis, Biological-Process (BP) analysis revealed that positive regulation of transcription, DNA-templated, oxidation-reduction process, G2/M transition of mitotic cell cycle, positive regulation of transcription from RNA polymerase II promoter, regulation of nitric-oxide synthase activity, ion transmembrane transport, response to drug, inflammatory response, positive regulation of cell proliferation, negative regulation of transcription from RNA polymerase II promoter were most significantly associated with Isorhamnetin action (Figure 5B); Cellular Component (CC) analysis indicated that the key terms were nucleus, cytoplasm, cytosol, nucleoplasm, plasma membrane, extracellular exosome, extracellular region, centrosome, perinuclear region of cytoplasm, Golgi apparatus (Figure 5C); Molecular Function (MF) analysis indicated that the top-ten enriched terms were protein binding, protein kinase binding, ATP binding, protein homodimerization activity, kinase activity, enzyme binding, protein serine/threonine kinase activity, transcription factor binding, nitric-oxide synthase regulator activity, ion channel binding (Figure 5D). For KEGG pathway analysis, the anti-cancer mechanisms of ISO included pathways in cancer, Estrogen signaling pathway, Dopaminergic synapse, Insulin signaling pathway, Progesterone-mediated oocyte maturation, Prostate cancer, Neurotrophin signaling pathway, cAMP signaling pathway, PI3K-Akt signaling pathway, Amphetamine addiction (Figure 5E).

## Isorhamnetin regulates GSK-3β/PI3K/AKT signaling pathway in liver cancer cells

According to the anti-tumor effect of ISO observed on MHCC97-H cells in the early research, combined with the KEGG signal pathway results predicted by PPI network analysis. Considered that PI3K/AKT signaling pathway was plays significant roles in modulating cell survival, proliferation, and apoptosis.

To further investigate the possibility of interaction between ISO and the key targets, we applied molecular docking studies [14]. The key targets could be analyzed by molecular docking after searching the RCSB Protein Data Bank database, and the main information are GSK3β (PDB id: 6h0u), CDK2 (PDB id:6q4g), NOS3(PDB id:3n5w), RELA (PDB id:6nv2), PIK3CG (PDB id:1e7u) and HSP90 (PDB id:6n8y). Based on the molecular docking criterion [14]: The ligands and receptors were considered to be able to form stable compounds when the binding energy was less than − 5 kcal/mol (Figure 6A), all the target genes from PI3K/Akt pathway predicted by PPI could bind to ISO well. Considering the compound has strong binding capacity with the target protein when the binding energy is < - 7 kcal/mol, we selected proteins with energy less than 7 for in-depth analysis (Figure

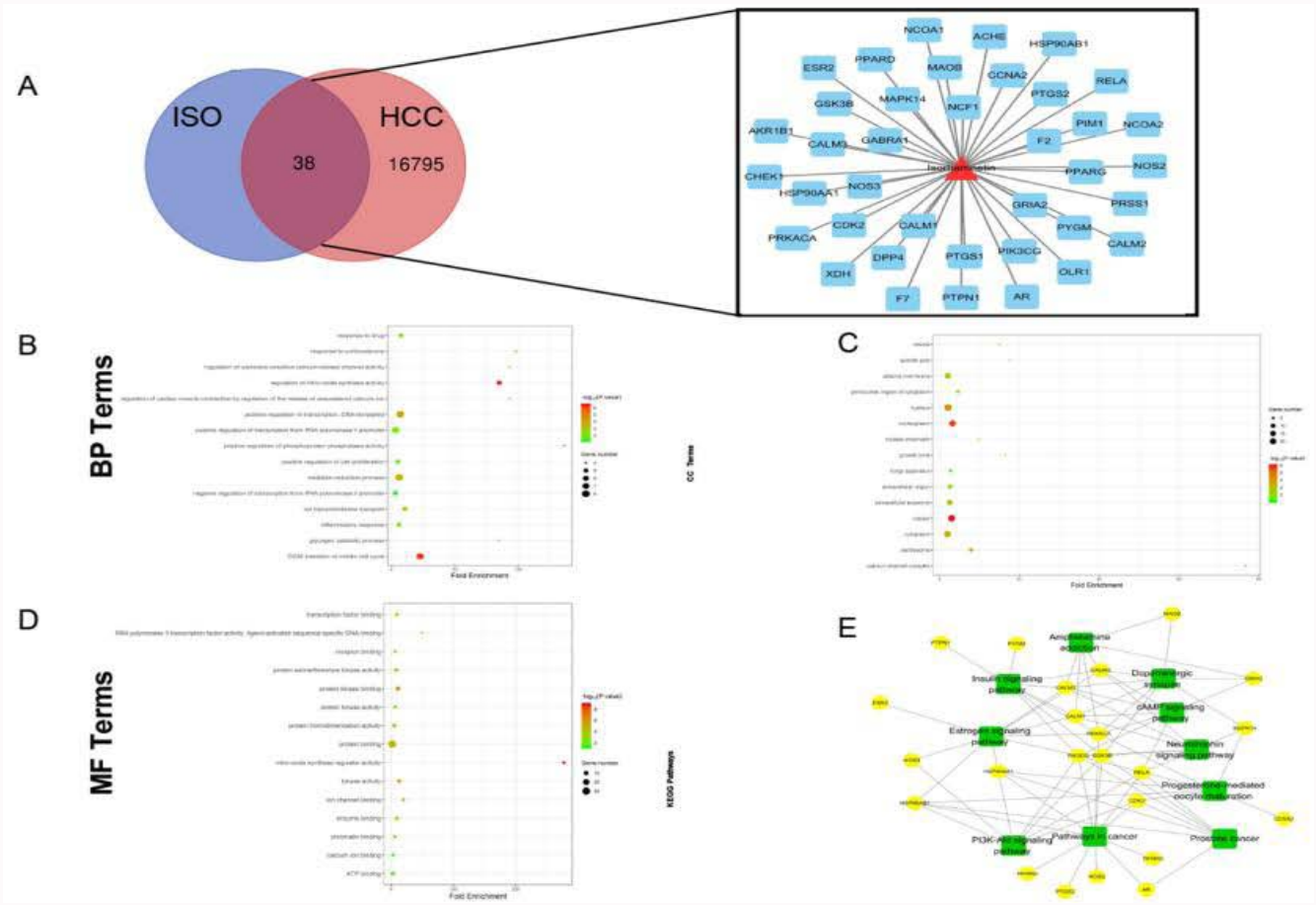


**Figure 5:** Establishment of an ingredient-target-hepatocellular carcinoma network of ISO. (A) The Venn analysis showed the target genes between ISO and hepatocellular carcinoma. (B) Bubble diagram showed the GO terms of Biological Processes (BPs), (C) Cellular Components (CCs), as well as (D) Molecular Functions (MFs), and (E) KEGG terms for enrichment analysis.

6B). The docking of the hub targets with ISO was mainly through conventional hydrogen bond (shown as yellow dotted line), carbon–hydrogen bond, covalent bond, and so on.

To determine whether ISO could regulate cell proliferation and apoptosis in HCC by activation of the GSK3β/PI3K/AKT signaling pathway. Results from Western blotting (Figure 6C, 6D) showed that after admission the ISO, the main protein expressions of NOS3 was increased, the expressions of p-PI3K, p-AKT, p-GSK3β, HSP90, CDK2 were significantly decreased, while the total GSK3β, AKT and PI3K did not change significantly after the administration of ISO at different concentrations in, which was highly similar to the molecular docking results.

### Isorhamnetin inhibits tumor growth in a MHCC-97H tumor xenograft model

Finally, the anticancer-like activity of ISO was evaluated in nude mice with xenografted tumor *in vivo*. The mice were subcutaneously injected with MHCC-97H cells, and then randomly assigned to four groups: (1) model; (2) ISO-5 mg/kg; (3) ISO-20 mg/kg; and (4) 5-Fu-50 mg/kg. After 15 days, the mice were sacrificed for tumor tissue collection and weight (Figure 7A, 7C). Treatment with ISO significantly reduced the weight of the xenografted tumors compared to the model. Consistently, the average tumor volume was significantly smaller in the ISO-20 mg/kg group compared with the control group from Day 5 to the end of the study (Figure 7B), while induced little weight loss throughout the entire experiment following the drug treatment (Figure 7E). Meanwhile, H&E staining and the Ki67 assay shown that ISO caused an increase in the apoptotic ratio and a decrease in ki67 expression (Figure 7D). After treatment of tumor bearing nude mice with 5-Fu or ISO, the tumor tissues showed trabecular and crumby structure, with tumor cells of round or oval shape, inflammatory cell infiltration in the edge of tumors, but a relatively mild and diffused necrosis. Ki67 staining demonstrated cancer cell nuclear proliferation level. 5-Fu or ISO admission markedly decreased the cell expression compared with model group. Further, western blotting detected the main protein about GSK3β/PI3K/AKT pathway, as the result showed that after admission the ISO, the main protein expressions of p-PI3K, p-AKT, p-GSK3β was inhibited, while the total GSK3β, AKT and PI3K did not change significantly (Figure 7F). These results indicate that ISO is a potential adjuvant drug for liver cancer treatment with good safety.

## Discussion

Natural products derived from plants have become an important source for drug development because their secondary metabolites are diverse and complex chemical components with good biological activity. Natural products were widespread attention in the field of anti-cancer treatment owing to their low toxicity, multiple targets, and curative effects [15], and the effects of ISO on HCC have not been widely studied. This study elucidated how ISO stimulates apoptosis,

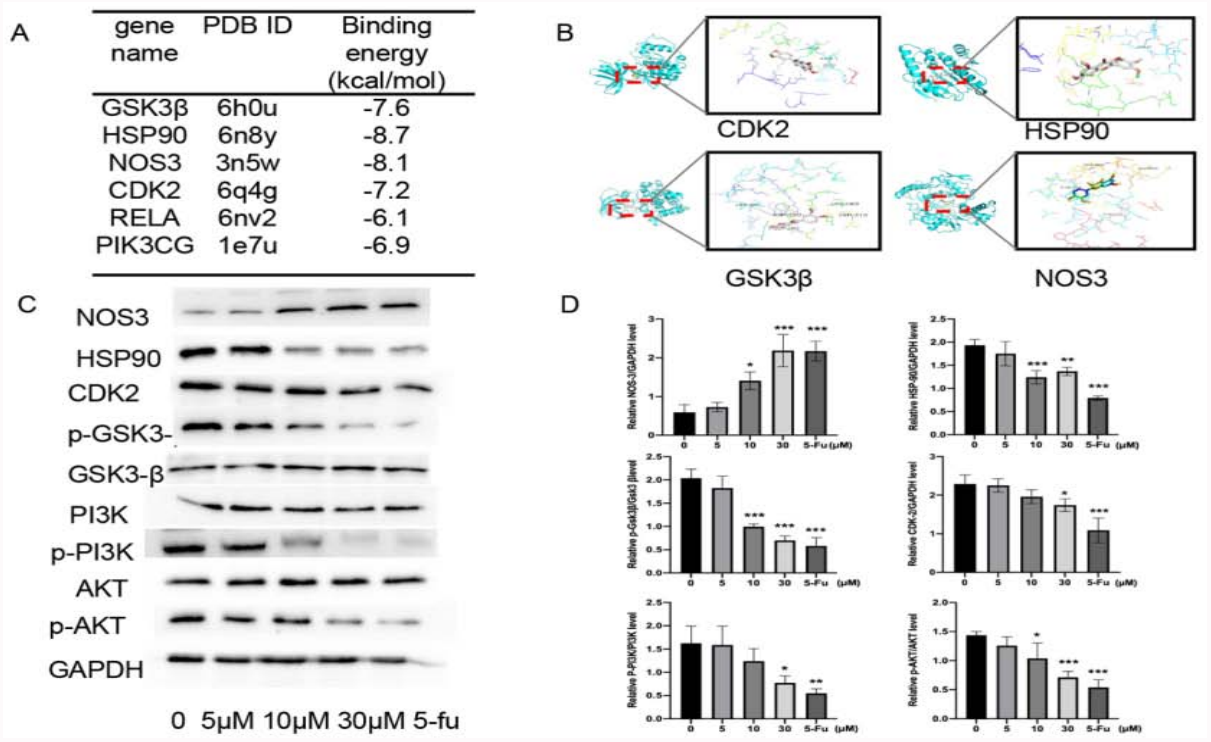


**Figure 6:** ISO regulated liver cancer in MHCC-97H cells through GSK3-β/PI3K/Akt Signaling Pathway. (A) Six proteins from PI3K/AKT signal pathway were predicted through PPI, and them molecular docking energy with ISO. (B) 3D structural simulation of main protein interacting with ISO. (C) Western blotting analysis the protein expression of the PI3K/AKT signaling pathways, protein expression of the NOS3, HSP90, CDK2, p-AKT, p-PI3K, p-GSK3β and the total protein expression of PI3K, AKT, GSK3β, quantification of protein expression was performed (D). The protein densitometry data were normalized with β-actin, and the p-GSK3β, p-AKT, p-PI3K, were normalized with the GSK3β, AKT, PI3K. All values represent the mean ± SD (*p<0.05, **p<0.01, ***p<0.001, *vs.* control, n=3).

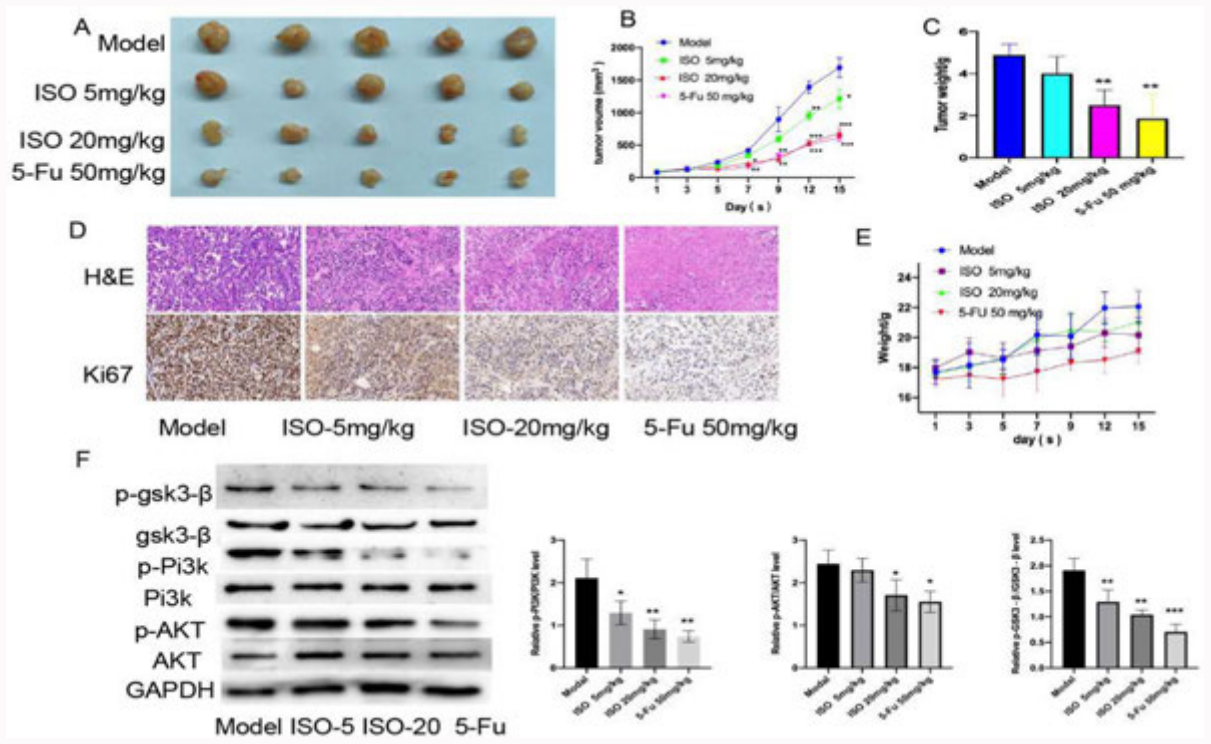


**Figure 7:** ISO inhibited tumor growth in MHCC-97H tumor xenograft models. A total of 20 mice were randomly divided into four groups and were treated with 0.9% normal saline (model group), ISO-5 mg/kg; and ISO-20 mg/kg; 5-Fu-50 mg/kg. (A) After 15 days, the mice were sacrificed for tumor tissue collection. (B) The tumor volume was measured every two days. (C) the tumors were weighted after 15 days. (D) H&E staining and IHC detection of Ki67 from the indicated groups. (E) The mice weight was measured and there was no significant decrease following drug treatment. (F) The protein expression of p-AKT, p-PI3K, p-GSK3β and the total protein expression of PI3K, AKT, GSK3β were measured by Western blotting. All values represent the mean ± SD (*p<0.05, **p<0.01, ***p<0.001, *vs.* control, n=3).

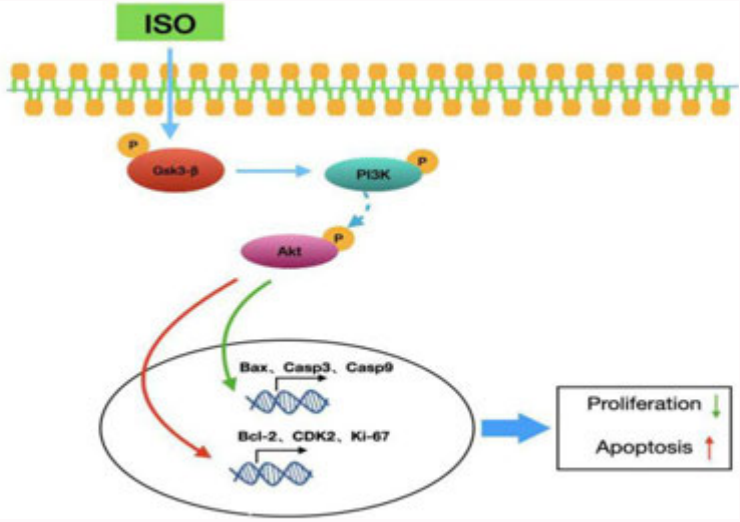


**Figure 8:** Proposed scheme for the protective effects of ISO on HCC. A summary of our current hypothesis for the mechanisms by which the ISO inhibit against cell proliferation and enhanced cell apoptosis in MHCC-97 cell. The protective effects of ISO may be associated with the activation of GSK3-β/PI3K/AKT signaling pathway.

and inhibits metastasis of liver cancer cells *in vitro* and *in vivo* in a dose-dependent approach (Figure 8).

In our study, the anti-proliferation properties of ISO were elevated as shown by CCK8 and colony formation assays on liver cancer cell lines HepG-2, MHCC-97H and normal liver cell LO-2. ISO has been identified to effectively suppress HepG-2 and MHCC-97H cells proliferation in a time- and dose-dependent manner, and no obvious influence on LO-2 cells. And the effect of cloning formation on MHCC-97H cell is much well. Cell migration plays a critical role in the establishment and maintenance of multicellular organisms [16]. It is required for the normal biological process such as wound healing, immune response and embryonic development [17]. Transwell and wound healing assays were found that ISO can significantly inhibit the migration and invasion capability on MHCC-97H cells. And apoptosis has become an important index to evaluate the effect of anti-cancer medicine [18,19]. Bcl-2 is considered to be a key molecule that inhibits apoptosis [20,21]. Bax, cleaved Caspase 9, and cleaved Caspase 3 are deemed to be key molecules that promote apoptosis [22]. A flow cytometric assay was performed to confirm that ISO inhibits MHCC-97H by inducing apoptosis. And western blot assay demonstrated that ISO significantly suppresses the expression of bcl-2 while it upregulated bax cleaved Caspase 9, and cleaved Caspase 3. Through a series of experiments, it was observed that ISO has obvious apoptosis-promoting effect on liver cancer cells.

The application of network pharmacology combined with molecular docking can enable further exploration of the potential molecular basis and mechanism of ISO in the treatment of HCC [23]. Bioinformatics analysis has further optimized the high-throughput screening strategy to validate candidate targets associated with diseases [24]. In this study, network and bioinformatics analysis revealed a total of 38 genes closely correlated with the ISO through Venn diagram analysis with liver cancer. Among these hub molecules, the top 20 genes including NOS3, NOS2, ESR2, AR, PPARG, PTPN1, MAPK14, GSK3B, HSP90AA1, HSP90AB1, CDK2, PIK3CG, DPP4, PRKACA, PIM1, CCNA2, NCOA2, PRSS1, PTGS1, PTGS2 were identified. Meanwhile, pathway enrichment analysis demonstrated that the main pathways included Pathways in cancer, cAMP signaling pathway, PI3K-AKT signaling pathway, and these pathways are mainly involved in physiological and pathological processes, such as regulation of nitric-oxide synthase activity, inflammatory response, cell cycle and apoptosis. *In vitro* experiments showed that ISO inhibited the proliferation, invasion and migration of HCCs, it's all proved that the effect of ISO on HCCs was more closely related to PI3K/AKT signaling pathway.

As everyone knows, anti-tumor effect about compounds mainly aims at the proliferation and apoptosis of tumor cells. Since GSK3β was found to plays oncogenic roles in multiple tumor types, there are research shown that nuclear GSK3β is responsible for the accumulation of the histone demethylase KDM1A and critically regulates histone H3K4 methylation during tumorigenesis [25]. Have effect on the development and progression of HCC [26]. Cancer cell proliferation is also a complex and ordered process that is rigorously regulated by cell cycle activity [27,28]. CDK2 as an important CDK family member, when CDK2 activated, the cell cycle was promoted enter to the S phase through the restriction point of G1/S, which controls the replication of DNA and centrosome, and can also promote the occurrence of mitosis during the G2/M transition [29].

The PI3K/AKT signaling is usually expressed highly in HCC and is believed to be conducive to the invasive phenotype and resistance to chemotherapies [30-32]. The PI3K/AKT signaling pathway plays essential roles in various aspects of cell growth and survival during tumorigenesis. Its activation could inhibit apoptosis and promote proliferation of cancer cells [30,32-34]. AKT is also known as Protein Kinase B (PKB), which is the main downstream effector of Phosphoinositide 3-Kinase (PI3K). Phosphorylated AKT (p-AKT) is involved in many physiological and pathological processes [13,35,36].

## Conclusion

In this study, we confirmed that Isorhamnetin inhibits tumor growth in a dose-dependent approach. Moreover, there was no substantial weight loss observed in the Isorhamnetin groups, indicating safety and the absence of side effects *in vivo*. We demonstrate that Isorhamnetin may suppress proliferation and metastasis, and triggering GSK3β/PI3K/AKT signaling cascade without any side

effects, which could provide a novel potential treatment strategy for HCC.

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