Research Square

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Integrating network pharmacology, bioinformatics, and experimental validation to unveil the molecular targets and mechanisms of galangin to treat hepatocellular carcinoma

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Abstract

Background Galangin, a flavonoid compound, is derived from *Alpinia officinarum* Hance. Previous studies have shown that galangin can inhibit the proliferation of hepatocellular carcinoma (HCC), but its mechanism is still unclear. This study aims to investigate the potential targets and molecular mechanisms of galangin on HCC through network pharmacology, bioinformatics, molecular docking, and experimental *in vitro* validation.

Methods In this study, network pharmacology was investigated firstly to determine the targets and mechanisms of galangin in the treatment of HCC. AutoDockTools software was used to further simulate and calculate the binding of galangin to core targets.GO and KEGG enrichment analyses were conducted in the DAVID database to explore the main biological functions and signaling pathways impacted by galangin intervention. Moreover, bioinformatics was used to analyze the correlation between the differential expressions of galangin's anti-HCC core targets and the survival of HCC patients. Finally, the findings obtained from network pharmacology and bioinformatics were verified in cell experiments.

Results A total of 67 overlapping target genes of galangin and HCC were identified. Through the PPI network analysis, 10 hub genes with the highest degree of freedom, including SRC, ESR1, MMP9, CDK4, CCNB1, MMP2, CDK2, CDK1, CHEK1, and PLK1, were screened and found to be closely related to the decomposition of the extracellular matrix, signal transduction, and the cell cycle, etc. GO and KEGG enrichment analyses revealed that galangin exerts an anti-HCC role by affecting various signaling pathways, including the cell cycle, pathways in cancer, and the PI3K-Akt signaling pathway, etc. Molecular docking demonstrated that CCNB1, CDK4, CDK1, and PLK1 had a powerful bond with galangin. Bioinformatics revealed that CCNB1, CDK4, CDK1, and PLK1 were up-regulated in the livers of patients with HCC at both mRNA and protein levels. The in *vitro* experiment showed that galangin can induce apoptosis of HepG2 and Huh7 cells and inhibit the proliferation of HCC cell. **Conclusions** Galangin induces apoptosis in HCC cells by blocking the cell cycle at the G0/G1 or G2/M phase through the inhibition of cyclins or mRNA expression.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. Patients with early-stage HCC who have had the tumor completely removed can anticipate positive treatment results. Nevertheless, the early symptoms of HCC are not easily noticeable, and when it is eventually diagnosed, it is usually in a late stage. Patients with advanced HCC who have missed the optimal period for surgical treatment are often only able to be treated with drugs. Although sorafenib, as the first-line drug in the treatment of advanced HCC, can significantly improve symptoms and control the disease, long-term use often causes serious adverse reactions such as diarrhea, gastrointestinal bleeding, and hypertension [1]. Therefore, it is the focus of current research to continuously explore therapeutic targets for HCC and develop highly efficient and specific targeted drugs for the treatment of HCC.

Evidence has shown that traditional Chinese medicine can be beneficial in alleviating symptoms and enhancing the quality of life of patients with HCC, inhibiting the recurrence of the tumor, and controlling the progression of the disease [2]. Galangin, a flavonoid derived from nature, is present in *Alpinia officinarum* Hance, *Plantago depressa* Willd., and propolis [3]. Numerous studies [4–6] have revealed that galangin has a comprehensive range of anti-tumor effects, such as colon cancer, breast cancer, leukemia, lung cancer, esophageal cancer, and hepatocellular carcinoma. Galangin can regulate the expression of cyclin D1, CDK4, CDK6, and other cell cycle-related proteins, inhibit the transformation of colon cancer cells from G0/G1 phase to S1 phase, and ultimately induce cancer cell cycle arrest [7]. Another study [8] showed that galangin can arrest the cell cycle of leukemia in G0/G1 phase, which may be related to regulating the expression of pRb, CDK4, CDK1 and cycline B, thus causing cell cycle arrest. In addition, galangin can also affect the expression of cyclin D3, cyclin E, and cyclin A, which makes human breast cancer cells be blocked in the G0/G1 phase, and finally plays a therapeutic role in breast cancer [9]. However, the mechanism of action of galangin against HCC through regulation of cycle-associated proteins has yet to be elucidated.

In the present study, network pharmacology was used to predict the potential targets and signaling pathways of galangin in the treatment of HCC. Then, molecular docking techniques were used to analyze the binding ability of galangin to the core targets, and bioinformatics was used to analyze the differential expression of the core targets in HCC. To ascertain the potential mechanism of galangin in the treatment of HCC, HepG2 and Huh7 cells were utilized as the study objects. This study found a new mechanism of action of galangin in the treatment of HCC, and provided a new idea and theoretical basis for the treatment of HCC with small-molecule compounds of traditional Chinese medicine.

Materials and methods

Prediction of potential targets for the treatment of HCC with galangin

The target of galangin predicted on the SwissTargetPrediction website was intersected with the target of galangin screened from the BATMAN-TCM database. After being corrected by the UniProt Knowledgebase, the drug targets of galangin were identified. The keyword "Hepatocarcinoma" and the genus limited to "Homo sapiens" were used to search the PharmGKB, DisGeNET, OMIM, and GeneCards databases. Then, using GeneCards score ≥ 15 and DisGeNET ≥ 0.10 as screening conditions, the disease targets of HCC were collected and the duplicates were deleted. The drug target of galangin and the disease target of HCC were intersected using Venny2.1 to obtain the anti-HCC target of galangin.

Construction of the intersection network and PPI network

With the species set as "Homo Sapiens" and the confidence level at 0.4, STRING11.5 was accessed to generate a PPI network map to explore the target of galangin in the treatment of HCC. Through the

analysis of key nodes in the PPI network with the Cytoscape3.9.1 software, core targets were identified according to the freedom value, thus constructing a "galangin-target-pathway-HCC" network.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment Analyses

By uploading the target of galangin to the DAVID database, with the species set to "Homo Sapiens" and the threshold set to $P < 0.05$, GO function and KEGG pathway Enrichment analysis can be conducted.

Molecular docking

The molecular structure of galangin was derived from the PubChem database. The selected core target protein molecular structure was downloaded from the PDB database and imported into the PyMol2.4.0 software for water and residue removal. The core target protein structure that had been processed was employed as the receptor, while the molecular structure of galangin acted as the ligand, which was then uploaded to AutoDocktools for further calculation and docking simulation. The docking data was finally visualized and analyzed using the PyMol software.

Bioinformatics analysis of the differential expression of core targets in HCC

The transcriptome data of the selected core genes in the present study were downloaded from the TCGA database. The mRNA expression differences of core targets in HCC human tissues were analyzed using the R language. CPTAC database was used to download the protein data of core targets and analyze their protein expression differences in HCC tissues through R language. The Kaplan-Meier plotter database was employed to examine the association between the expression of the core target genes and the survival of HCC patients.

Cell culture and treatment

The human hepatoma cell lines HepG2 and Huh-7, as well as human hepatic stellate cell line LX-2, were purchased from Bena Culture collection (BNCC, Suzhou, China). HepG2 cells were cultured in MEM medium, Huh-7 cells in RPMI 1640, and LX-2 cells in RPMI 1640. All media were supplemented with 10% FBS (v/v) and 1% penicillin-streptomycin, and kept in a humid atmosphere with 5% CO_2 at 37°C.

Cell viability assay

A density of 1×10^4 cells per well was seeded into 96-well plates, with 5 replicates per group, and incubated at 37℃ and 5% CO_2 for 12 hours. After 12 hours of medication, the liquid in the 96-well plates was discarded. Next, all wells were supplemented with 100 μL of medium containing CCK8 and incubated at 37℃ and 5% CO_2 for 1 hour. After incubation, the absorbance was measured at 450 nm with an enzyme marker.

Cell proliferation assays

Logarithmic growth stage cells were subjected to 0, 40, 80, and 120 μM concentrations of galangin. Following a 12-h incubation period, each well was exposed to a 2x EdU working solution at 37°C and 5% CO_2. Then, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.25%Triton X-100 for 15 min. The cells were incubated with the Click Additive Solution for 30 min and Hoechst for 10 min, respectively, and then observed under a fluorescence microscope.

Cell apoptosis and cell cycle analysis

Cells from various treatment groups were transferred into clean flow tubes, and Annexin V-FITC and PI staining were added to each tube, respectively, according to the instructions of the apoptosis kit. The tubes were left in darkness for 10–15 min before the cells were combined with Annexin V 1x Binding Buffer and put on ice. The apoptosis rate of each group was determined using flow cytometry. Cells from different treatment groups were fixed with 80% ethanol, and the PI staining solution was added in line with the cycle kit instructions.

Western blotting analysis

Cell samples from different treatment groups were cleaved with cell lysate, and the protein concentrations were determined according to the procedure of the BCA kit. The same amount of protein extract was subjected by 10% SDS-PAGE and then transferred to a PVDF membrane. The membrane with the protein was enclosed in 5% skim milk powder. After washing, the membranes were left to incubate with primary antibodies at 4 ℃ overnight. The next day, after washing the membrane, the second antibody was incubated at room temperature. After ECL color development, high sensitivity chemiluminescence imaging system was used for exposure development. The band intensity was quantified using Image Lab image processing software.

Quantitative real-time PCR (qPCR)

The total RNA of cells was obtained through Trizol reagent extraction, and its concentration was determined using Nano Drop One. Then, the total RNA was reverse transcribed into cDNA and amplified by PCR. The mRNA level was normalized to the endogenous housekeeping gene GAPDH and relative to the calibrator sample using the $2^{-\Delta\Delta Ct}$ method. The analyzed genes and their corresponding primer sequences are listed in Table 1.

Table 1
Primer sequences

Gene	Sequence (5’-3’)	
ATR	F: TTGTCTGGGCTCCCTTC	R: GCACAGCATCCCTGTTTT
CHK1	F: GTCACAGGAGAGAAGGCAA	R: AGCATCTGGTTCAGGCA
CDC25C	F: CGAGCGGGGATAGGTTA	R: CTGAGCAGAAGGCCAAAG
CCNB1	F: CTTTCGCCTGAGCCTATTT	R: CCATCTTCTGCATCCACAT
CDK4	F: AAGGAAGAAAAGCTGCCAT	R: TCAAAAGGAGAGGTGGGA
CDK1	F: AAAGTGAAGAGGAAGGGGTT	R: TGTACTGACCAGGAGGGATAG
PLK1	F: TGCACCGAAACCGAGTTA	R: CCACACAGGGTCTTCTTCC
GAPDH	F: GGGGCTCTCCAGAACATC	R: TGACACGTTGGCAGTGG

Statistical analysis

All the experimental data were expressed as mean ± SD. Statistical analyses were conducted using GraphPad Prism 9.0. Data conforming to normal distribution were analyzed by one-way ANOVA. The results of homogeneity of variance were compared between groups using the LSD test. Dunnett's T3 test was used for unequal variances.

Results

Network pharmacology analysis of galangin

As shown in Fig. 1A, the chemical structural formula of galangin was derived from the TCMSP database. Through the BATMAN-TCM database and the SwissTargetPrediction website, 120 potential targets of galangin were identified. HCC disease targets were collected from various databases, including 1981 in the GeneCards database, 507 in the OMIM database, 1396 in the DisGeNET database, and 284 in the PharmGKB database. After merging and deleting duplicate targets, a total of 3319 HCC targets were collected (Fig. 1B). By intersecting 120 galangin targets and 3319 HCC disease targets, 67 shared targets of galangin against HCC were determined (Fig. 1C).

The target of galangin against HCC was input into the STRING database for processing, and a PPI network diagram was obtained (Fig. 1D). The relevant data were imported into Cytoscape 3.9.1 software, and network topology analysis was performed in the software to screen the top ten core targets with the highest degree of freedom (Fig. 1E). These targets include SRC, ESR1, MMP9, CDK4, CCNB1, MMP2, CDK2, CDK1, CHEK1, and PLK1.

67 common targets of galangin and HCC were analyzed for GO and KEGG enrichment. GO functional enrichment analysis showed that the target proteins were involved in 55 biological processes (BP), 44 cellular components (CC), and 41 molecular functions (MF). The top 10 terms according to gene counts in every category are presented as column charts (Fig. 1G). KEGG enrichment results showed that galangin can mainly affect 37 signaling pathways, including the cell cycle, pathways in cancer, the PI3K-Akt signaling pathway, microRNAs in cancer, and chemical carcinogenesis - receptor activation. The top 20 pathways enriched by KEGG were shown in Fig. 1H.

The 'Galangin - Target - Pathway - HCC' interaction network (Fig. 1F) was constructed through the importation of information files of galangin, common targets, signaling pathways, and HCC into Cytoscape 3.9.1. The network consists of 89 nodes and 322 edges. The yellow circle represents the target, the blue diamond indicates the signaling pathway, the red inverted triangle denotes galangin, the green triangle represents HCC, and the lines represent the interconnections between them. Galangin is associated with the cell cycle signaling pathway (hsa04110) by acting on core targets including CCNB1, CDK4, CDK1, and PLK1.

Molecular docking analysis of galangin

When the absolute value of the binding energy between the ligand and the receptor is greater, the binding is more stable. A binding energy of less than − 5 kcal/mol implies a strong affinity between the ligand and receptor [10]. Docking experiments were conducted, using galangin as a ligand, with four key targets: CCNB1, CDK4, CDK1, and PLK1, as receptors. As shown in the Fig. 2, the docking results of galangin and its key targets were visualized using the PyMol software. The binding energies of galangin with CCNB1, CDK4, CDK1, and PLK1 were − 5.4 kcal/mol, -5.5 kcal/mol, -7.8 kcal/mol, and − 5.6 kcal/mol, respectively, indicating that galangin has good binding affinity with each of these key receptors.

Bioinformatics analysis of galangin

In this study, the mRNA expressions of CCNB1, CDK4, CDK1, and PLK1 in liver tissues were analyzed using the TCGA database. As shown in Fig. 3A, C, E, & G, the mRNA expression levels of CCNB1 (P = 2.66e-28), CDK4 (P = 2.494e-18), CDK1 (P = 6.273e-28), and PLK1 (P = 1.584e-27) in the HCC group were significantly higher than those in the normal group. To further clarify the expression of CCNB1, CDK4, CDK1, and PLK1 at the protein level in the liver, high-throughput proteomic data based on mass spectrometry technology were analyzed in the CPTAC database. As shown in Fig. 3B, D, F, & H, compared with normal group, the expressions of CCNB1 (P = 3.291E-08), CDK4 (P = 5.258E-06), CDK1 (P = 3.744E-39) and PLK1 (P = 4.172E-04) proteins in HCC patients were up-regulated. The above results indicated that CCNB1, CDK4, CDK1, and PLK1 were up-regulated in the livers of HCC patients at both mRNA and protein levels.

The effects of mRNA expression of CCNB1, CDK4, CDK1, and PLK1 on the survival of HCC patients were evaluated using the TCGA database. The median mRNA expression levels of CCNB1, CDK4, CDK1, and PLK1 were used as cut-off values to divide HCC patients into high-expression and low-expression groups.

Kaplan-Meier survival analysis showed that the overall survival rate of HCC patients in the high mRNA expression group of CCNB1 (P = 0.00026), CDK4 (P = 0.00016), CDK1 (P = 0.0029), and PLK1 (P = 0.00036) was significantly lower than that of those with low mRNA expression group (Fig. 4A-D). Furthermore, the relationship between the expression levels of CCNB1, CDK4, CDK1, and PLK1 proteins in the liver and the survival of HCC patients was validated using the CPTAC database. Compared with the low-expression group of HCC patients, the overall survival rate of HCC patients with high expression levels of CCNB1 (P = 0.015), CDK4 (P = 0.023), CDK1 (P = 0.00081), and PLK1 (P = 0.00074) proteins was lower (Fig. 4E-H). The Kaplan-Meier Plotter online database was utilized to ascertain the correlation between the mRNA expression levels of CCNB1, CDK4, CDK1 and PLK1 and the survival rate of HCC patients. The results showed that the mRNA expression levels of CCNB1 (P = 0.00015), CDK4 (P = 0.0021), CDK1 (P = 0.00017), and PLK1 (P = 0.00082) were negatively correlated with the overall survival rate of HCC patients (Fig. 4I-L), which were consistent with the analysis results that were obtained from the TCGA database.

Galangin suppressed the viability and proliferation of HCC cells

As shown in the Fig. 5A, galangin had no inhibitory effect on the proliferation of LX-2 cells at concentrations of 40–160 μM for 12 h (P > 0.05). On the contrary, galangin showed a significant inhibitory effect on the proliferation of HepG2 cells (P < 0.001) and Huh7 cells (P < 0.05) (Fig. 5B & C). The results showed that galangin was cytotoxic to hepatocellular carcinoma cells, including HepG2 and Huh7 cells, in the range of 40–160μM, and could inhibit the proliferation of these two cancer cells, but not be cytotoxic to the normal LX-2 cell. The half maximal inhibitory concentration (IC50) of galangin on HepG2 and Huh7 cells was 73.88 μM and 82.41 μM, respectively (Fig. 5D & E). Therefore, concentrations of 40, 80, and 120 μM of galangin were selected for follow-up experiments.

Galangin inhibited cell cycle progression and induced apoptosis of HCC cell

As shown in the Fig. 6A & B, after treatment of HepG2 cells with different concentrations of galangin (40, 80, 120μM), flow cytometry showed that 80μM (P < 0.01), 120μM (P < 0.001) of galangin significantly increased the apoptosis rate of HepG2 cells. Meanwhile, galangin can induce Huh7 cell apoptosis in a dose-dependent manner (P < 0.001). These results indicated that galangin can induce apoptosis of HepG2 and Huh7 cells and inhibit the proliferation of HCC cell.

The effect of galangin on cell cycle of HepG2 cells was further detected by flow cytometry. HepG2 and Huh7 cells were treated with different concentrations of galangin (40, 80, 120 μM) for 12 h. As shown in the Fig. 6C, compared with the control group, the percentage of HepG2 cells in G0/G1 phase was dramatically increased in the galangin administration group (P < 0.001), and the percentage of HepG2 cells in S phase was significantly decreased (P < 0.001). Similarly, the percentage of Huh7 cells in G0/G1

phase was significantly increased in galangin administration groups ($P < 0.05$), while the percentage of Huh7 cells in G2/M phase was significantly decreased ($P < 0.05$) (Fig. 6D). The results of cell cycle experiment showed that galangin could block the cell cycle progression of HepG2 and Huh7 and inhibit the proliferation of HCC cell.

Galangin down-regulated the expression of the core targets

As shown in the Fig. 7A & B, Western Blot was used to detect the expression of CCNB1, CDK4, CDK1, and PLK1 proteins in HepG2 and Huh7 cells treated with different concentrations of galangin (40, 80, and 120 μM) for 12 h. Compared with the control group, the expressions of CCNB1, CDK4, CDK1 and PLK1 in HepG2 cells were significantly decreased ($P < 0.05$ or $P < 0.01$ or $P < 0.001$) in a dose-dependent manner. Similarly, galangin also inhibited the expression of CCNB1, CDK4, CDK1, and PLK1 in Huh7 cells in a dose-dependent manner ($P < 0.05$ or $P < 0.01$ or $P < 0.001$). CDC25C, a dual-specificity phosphatase, is essential for controlling the serine/threonine kinases that are involved in the cell cycle. Our experiments found that galangin could significantly downregulate the expression level of CDC25C protein in HepG2 and Huh7 cells.

In order to further study the effect of galangin on cell cycle-related targets, qRT-PCR was used to detect the mRNA expression of different concentrations of galangin on the relevant targets. As shown in the Fig. 7C & D, the mRNA expressions of ATR, CHK1, CDC25C, CCNB1, CDK4, CDK1, and PLK1 were significantly down-regulated in HepG2 and Huh7 cells treated with different concentrations of galangin. These results further corroborated that galangin is capable of modulating the core targets of the cell cycle by suppressing the ATR/Chk1 DNA damage response signaling pathway, thereby exhibiting anti-HCC activity.

Discussion

HCC is one of the most prevalent cancers in the world. With a low survival rate, this disease stands second only to pancreatic cancer in terms of five-year survival rate [11]. The chemoprevention method using small-molecule compounds from food sources has gradually become an important aspect of HCC treatment and control [12]. Galangin is a natural flavonoid that has anti-tumor effects on breast, liver, stomach, cervical, colorectal, lung, and bone tumors [13–16]. Although galangin has been extensively studied as an antineoplastic agent in various cancers, the mechanism of action of galangin in HCC remains unclear. Therefore, this study adopted network pharmacology methods to predict the potential targets and molecular mechanisms of galangin in the treatment of HCC, and analyzed the possibility of galangin binding to the targets of HCC through molecular docking technology. Additionally, bioinformatics was used to analysis the relationship between the targets and the occurrence and development of HCC.

In this study, network pharmacology and bioinformatics methods were used to predict and analyze the possible mechanism of galangin in regulating the cell cycle signaling pathways through targeting CCNB1,

CDK4, CDK1, and PLK1, thereby exerting its therapeutic effect on HCC. The cell-cycle signaling pathway is a common activation pathway in cancer development, which can regulate tumor cell apoptosis and the cell cycle [17]. Studies have shown that CCNB1 and CDK1 are highly expressed in HCC patients. However, knockdown of the CCNB1 and CDK1 genes by siRNA can significantly induce autophagy and senescence in HCC cells [18]. The combination of CCNB1 and CDK1 inhibitors with immunotherapy has been shown to increase the survival time of HCC patients [19]. CDK4 is the initial cyclin-dependent protein kinase to be activated when cells enter the proliferation cycle, and its activation state is the key to whether cells can enter the proliferation cycle. CDK4 inhibitors inhibit the proliferation of human HCC cell lines by promoting reversible cell cycle arrest [20]. PLK1 is a significant kinase in the control of the cell cycle and is abundantly expressed in HepG2 cells. Studies have shown that knocking down the PLK1 gene can significantly inhibit the migration ability of human liver cancer vascular endothelial cells [21, 22]. After 12 h of HepG2 and Huh7 cells being exposed to different concentrations of galangin, the proteins and mRNA expression levels of CCNB1, CDK4, CDK1, and PLK1 were significantly reduced, indicating that galangin can inhibit the cell cycle of HCC. These results were consistent with those of network pharmacological and bioinformatics analyses. At the same time, the results of molecular docking also showed that galangin had strong binding force with these targets.

Over the course of evolution, cells have established a rigorous and efficient set of checkpoint mechanisms to guarantee that their genetic material is accurately replicated and transmitted to subsequent generations [23]. DNA damage activates the cell cycle checkpoints, which stop the cell cycle, giving the DNA enough time to repair, or causing the cell to die if the damage is irreparable. From the end of G1 phase to S phase and G2 phase, the control of cell cycle is mainly mediated by the ATR/Chk1/CDC25 pathway. After DNA damage occurs, ATR accumulates at the site of the damage and is activated, followed by the activation of its downstream Chk1, which then activates p53, resulting in cell cycle arrest. Cell division cycle-25C (CDC25C) plays an important role in regulating the activity of serine/threonine kinases involved in the cell cycle. CDC25C can activate the CCNB1/CDK1 complex by triggering the dephosphorylation of cyclin-dependent kinase-1 (CDK1), thereby promoting the G2/M transition in mitotic cells. In this experiment, PCR was employed to assess the expression of ATR, Chk1, and CDC25C mRNA in HepG2 and Huh7 cells following treatment with galangin. The results showed that galangin effectively reduced the expression of ATR, Chk1, and CDC25C mRNA in HCC cells. The cell cycle signaling pathway interfered with by galangin is shown in Fig. 8. The findings of the cell cycle study demonstrated that galangin inhibited HepG2 cells in the G0/G1 phase and Huh7 cells in the G2/M phase.

Conclusions

In summary, galangin induced apoptosis in HCC cells by blocking the cell cycle at the G0/G1 or G2/M phase through the inhibition of cyclins or mRNA expression. This study provides a new research direction for the treatment of HCC by galangin, and provides ideas and theoretical basis for the study of lead compounds for the treatment of HCC.

Abbreviations

HCC, Hepatocellular carcinoma

qPCR, Quantitative real-time PCR

BP, biological processes

CC, cellular components

MF, molecular functions

CDC25C, Cell division cycle-25C

GO, Gene Ontology

KEGG, Kyoto Encyclopedia of Genes and Genomes

CDK1, cyclin-dependent kinase-1

Declarations

Author contributions

Jian Xu, Junqing Zhang, and Yipeng Pan provided fund support and designed this experiment. Xiaoliang Li, Jiangbo Sun, Bingshu Wang, Yihang Zhao, and Gaoan Wang carried out this experiment and wrote the article. Mingyan Zhou and Weijia Chen provided assistance in conducting experiments and performing statistical analysis. All authors read and approved the final manuscript.

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Data availability

The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

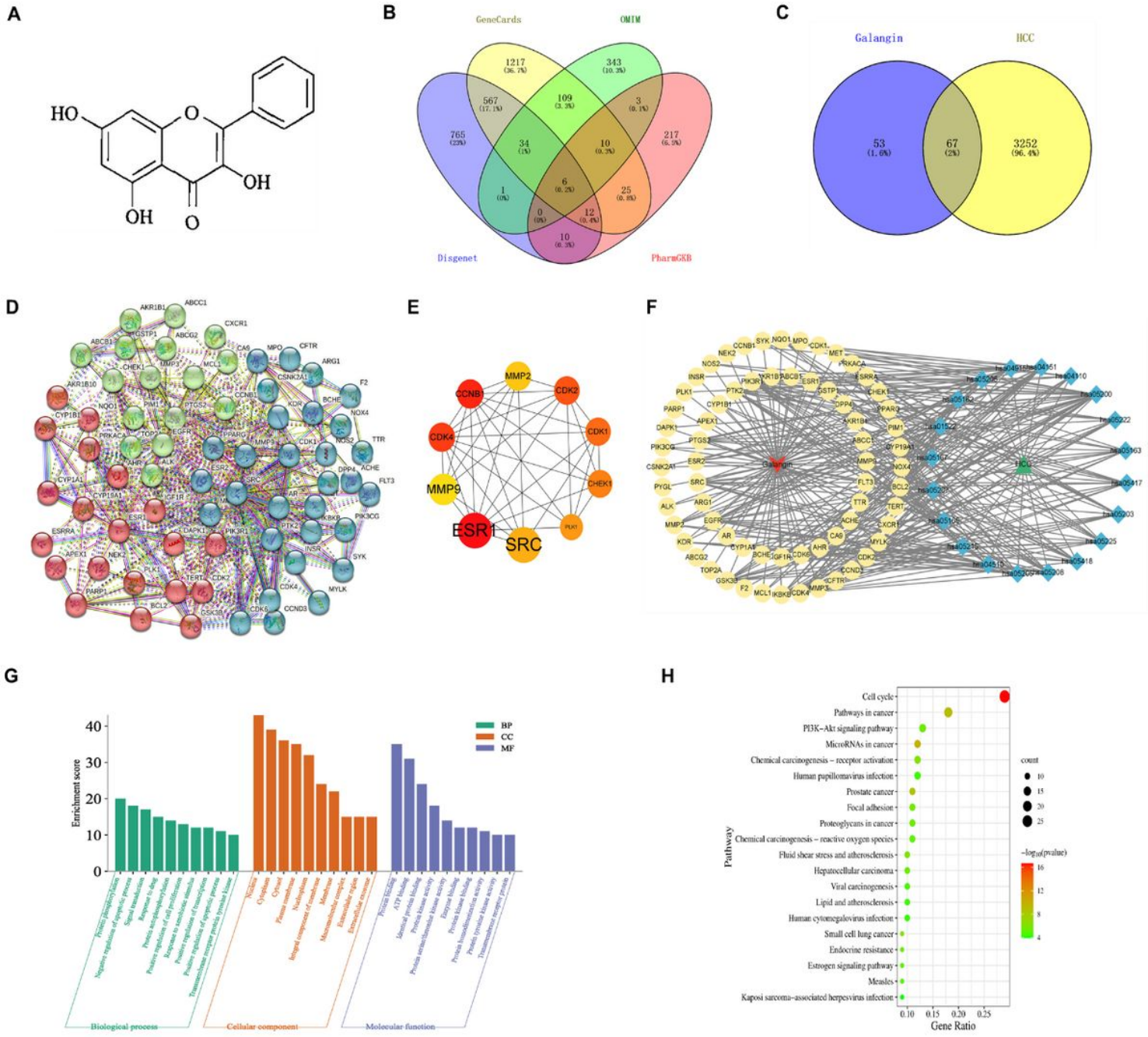


Figure 1

Network pharmacology predicted potential mechanisms of galangin for HCC. **(A)** Chemical structure formula of galangin. **(B)** Venn diagram of HCC-related targets in GeneCards, OMIM, DisGeNET, and PharmGKB databases. **(C)** Venn diagram of targets shared by galangin and HCC. **(D)** The top ten core targets of degree of freedom values in topological analysis. **(E)** Protein-protein Interaction (PPI) network. **(F)** Network diagram of galangin -pathway-HCC-target. **(G)** Top 10 terms of biological process, cellular component, and molecular function enrichment analysis from GO enrichment analysis. **(H)** KEGG enrichment analysis of potential therapeutic targets of galangin against HCC

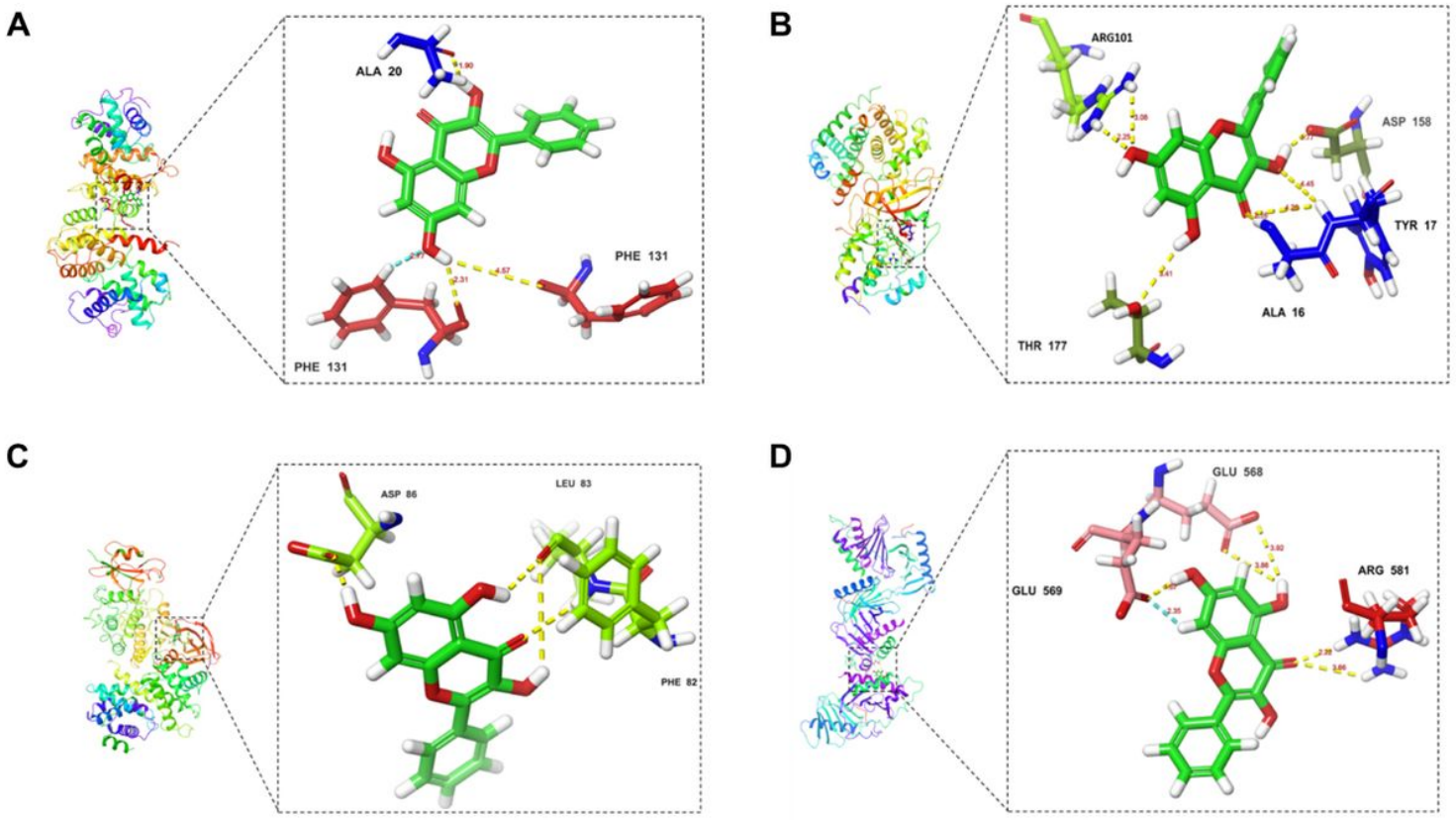


Figure 2

Validation of interactions between galangin and HCC. **(A)** Docking sites of CCNB1and galangin. **(B)** Docking sites of CDK4 and galangin. **(C)** Docking sites of CDK1 and galangin.**(D)** Docking sites of PLK1and galangin

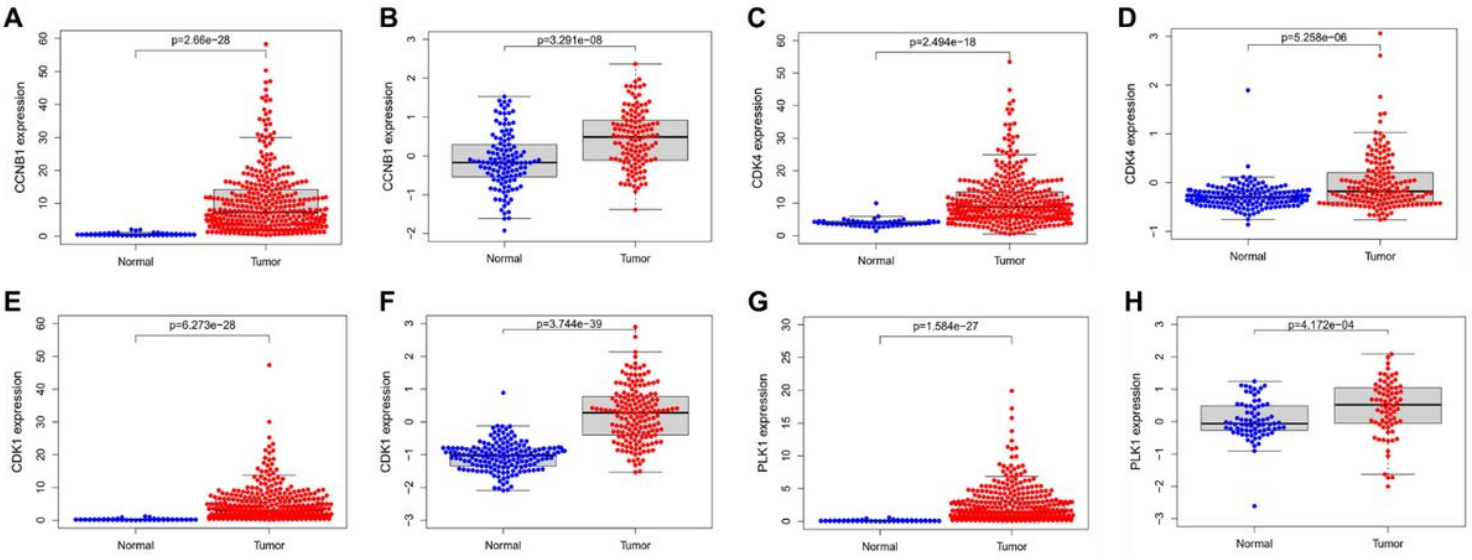


Figure 3

Differential expression of core targets in liver tissues. **(A)** Differential expression of CCNB1 mRNA in the liver. **(B)** Differential expression of CCNB1 protein in the liver. **(C)** Differential expression of CDK4 mRNA in the liver. **(D)** Differential expression of CDK4 protein in the liver. **(E)**Differential expression of CDK1 mRNA in the liver. **(F)** Differential expression of CDK1 protein in the liver. **(G)** Differential expression of PLK1 mRNA in the liver. **(H)** Differential expression of PLK1 protein in the liver

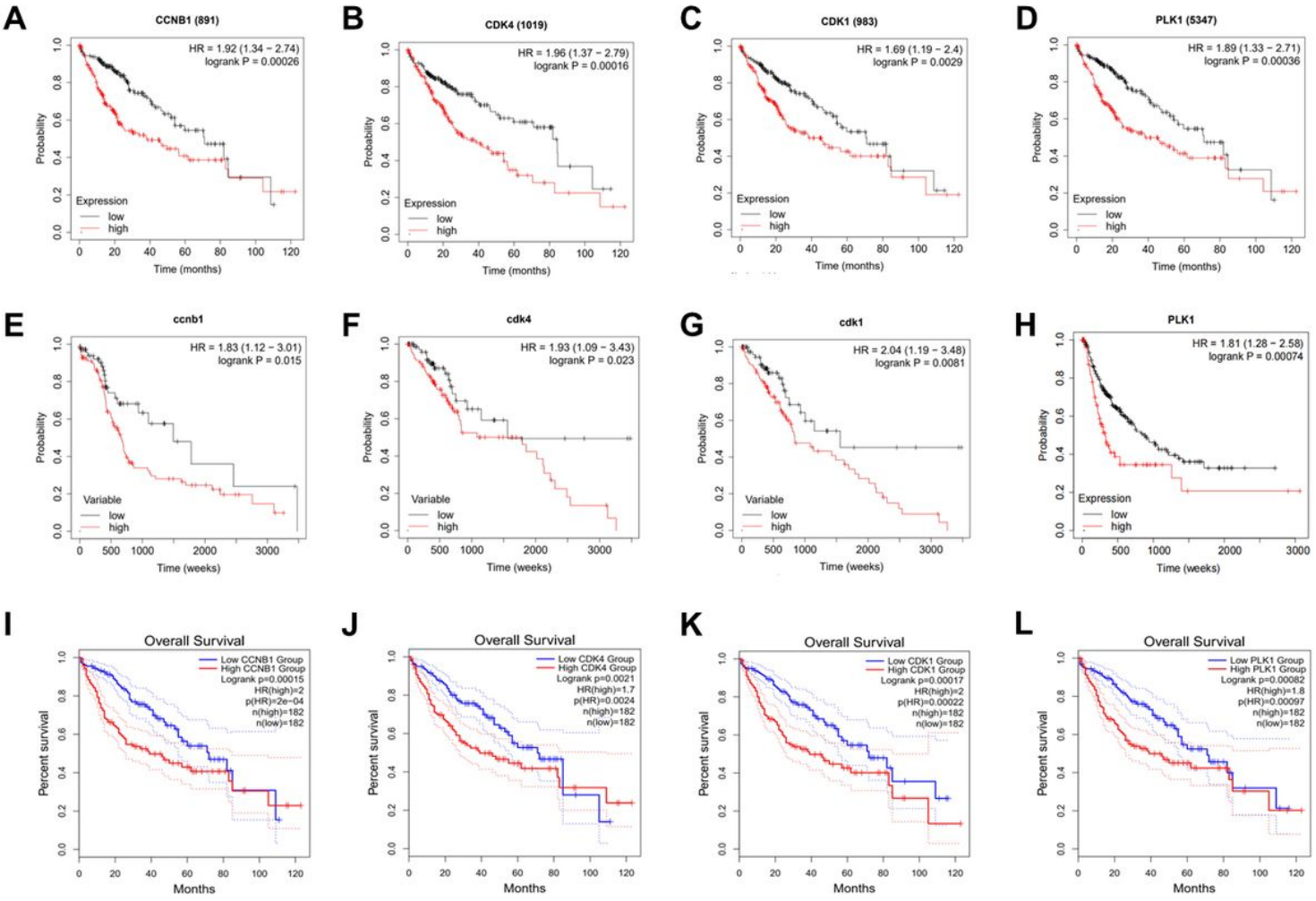


Figure 4

Relationship between core target expression and overall survival in HCC patients. The effect of CCNB1 **(A)**, CDK4 **(B)**, CDK1 **(C)**, and PLK1 **(D)** mRNA expression levels on the survival of HCC patients was evaluated using the TCGA database. The effect of CCNB1 **(E)**, CDK4 **(F)**, CDK1 **(G)**, and PLK1 **(H)** protein expression levels on the survival of HCC patients was evaluated using the CPTAC database. The effect of CCNB1 **(I)**, CDK4 **(J)**, CDK1 **(K)**, and PLK1 **(L)** mRNA expression levels on the survival of HCC patients was further confirmed by the Kaplan-Meier plotter online database.

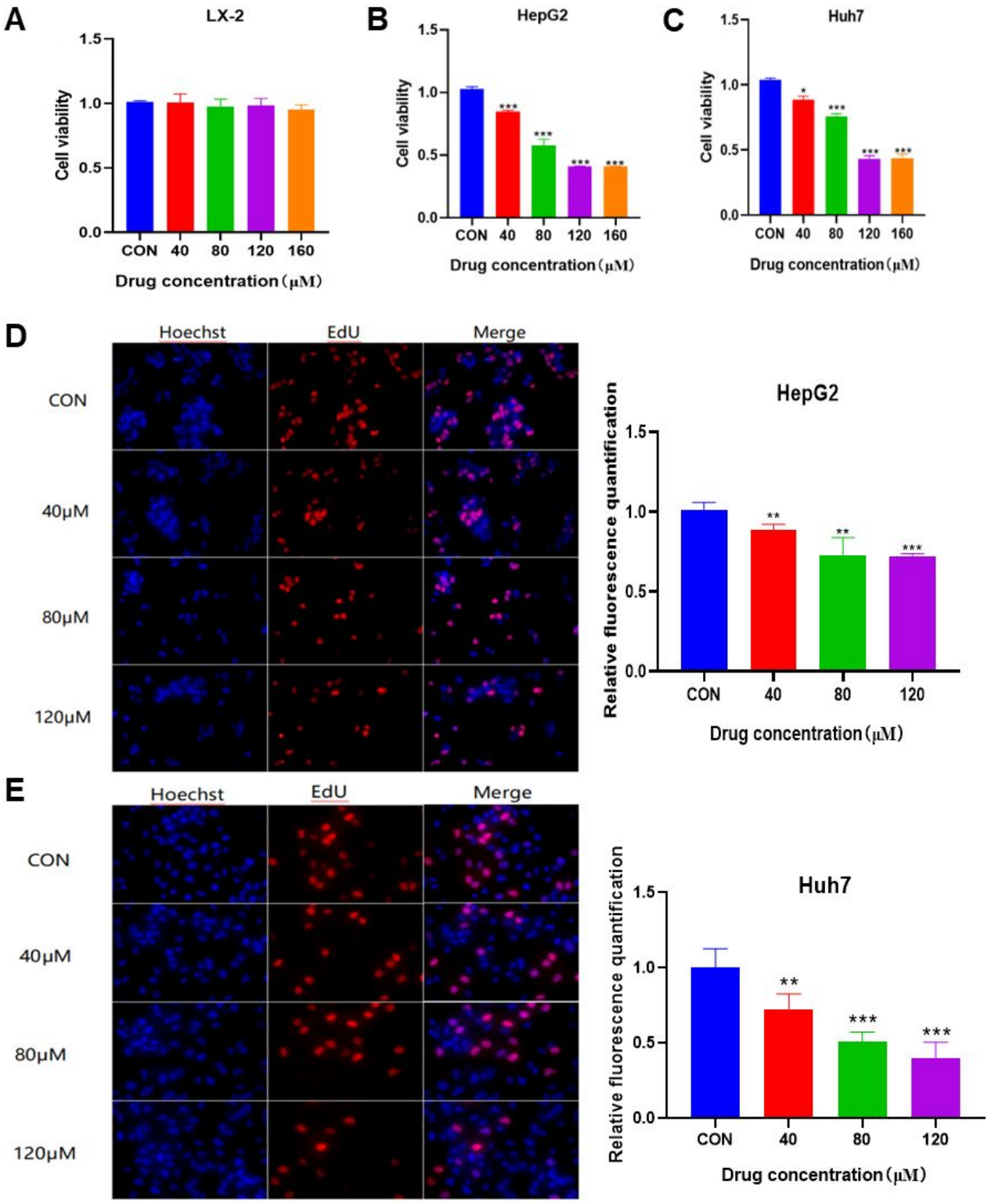


Figure 5

Effect of galangin on viability and proliferation of three cell lines. The cytotoxicity of galangin (40, 80, 120, and 160μM) on **(A)** LX-2, **(B)** HepG2, and **(C)** Huh7 cells was determined by the CCK8 method. Effect of galangin (40, 80, and 120 μM) on the proliferation of **(D)** HepG2 cells and **(E)** Huh7 cells. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

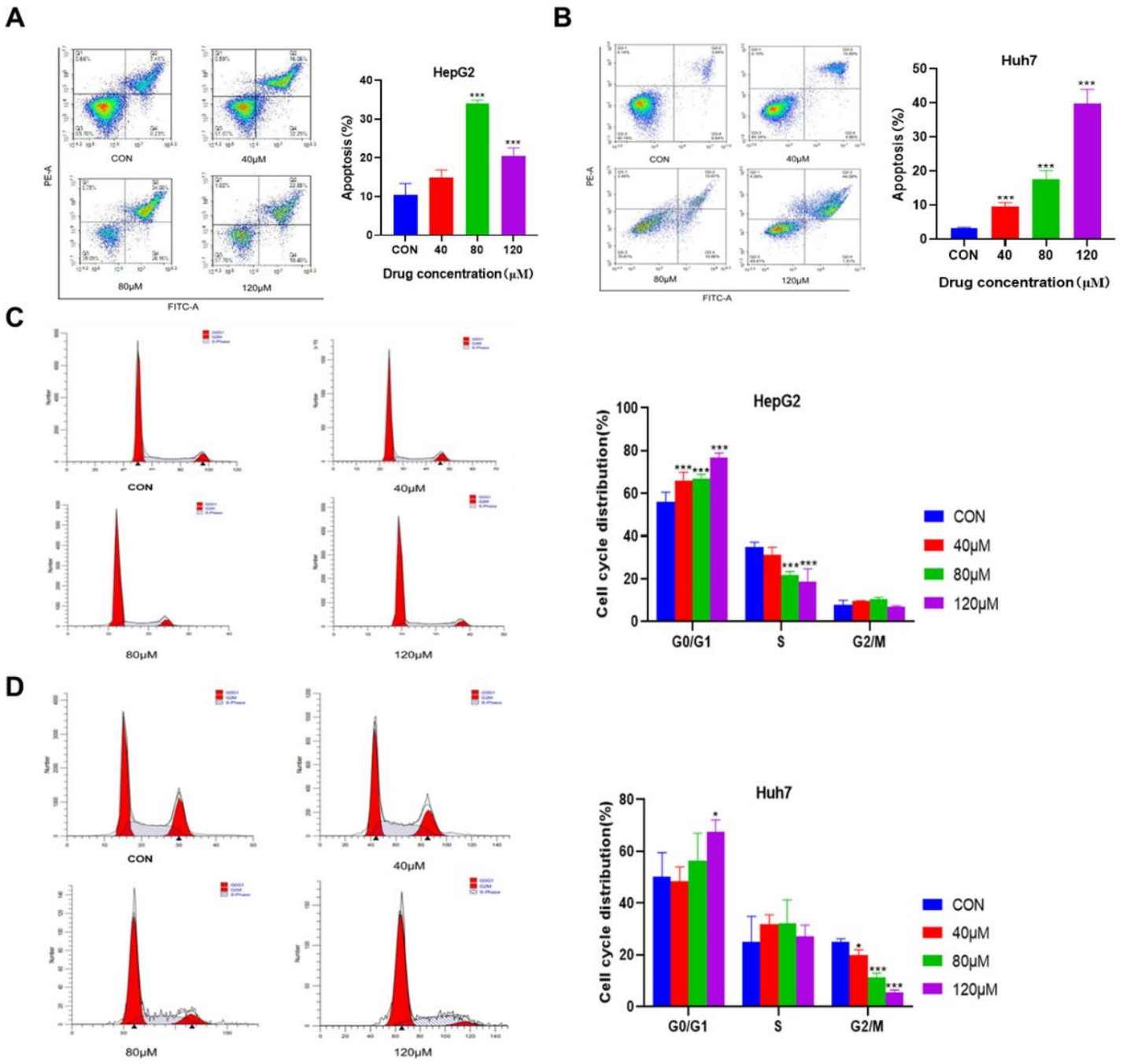


Figure 6

Effects of galangin treatments on HCC cell apoptosis and cell cycle progression. The apoptosis of HepG2 **(A)** and Huh7 cells **(B)** was evaluated using flow cytometry. The cell cycle progression of **(C)** HepG2 and **(D)** Huh7 cells was measured by flow cytometry. $^{**}P<0.01$, $^{***}P<0.001$.

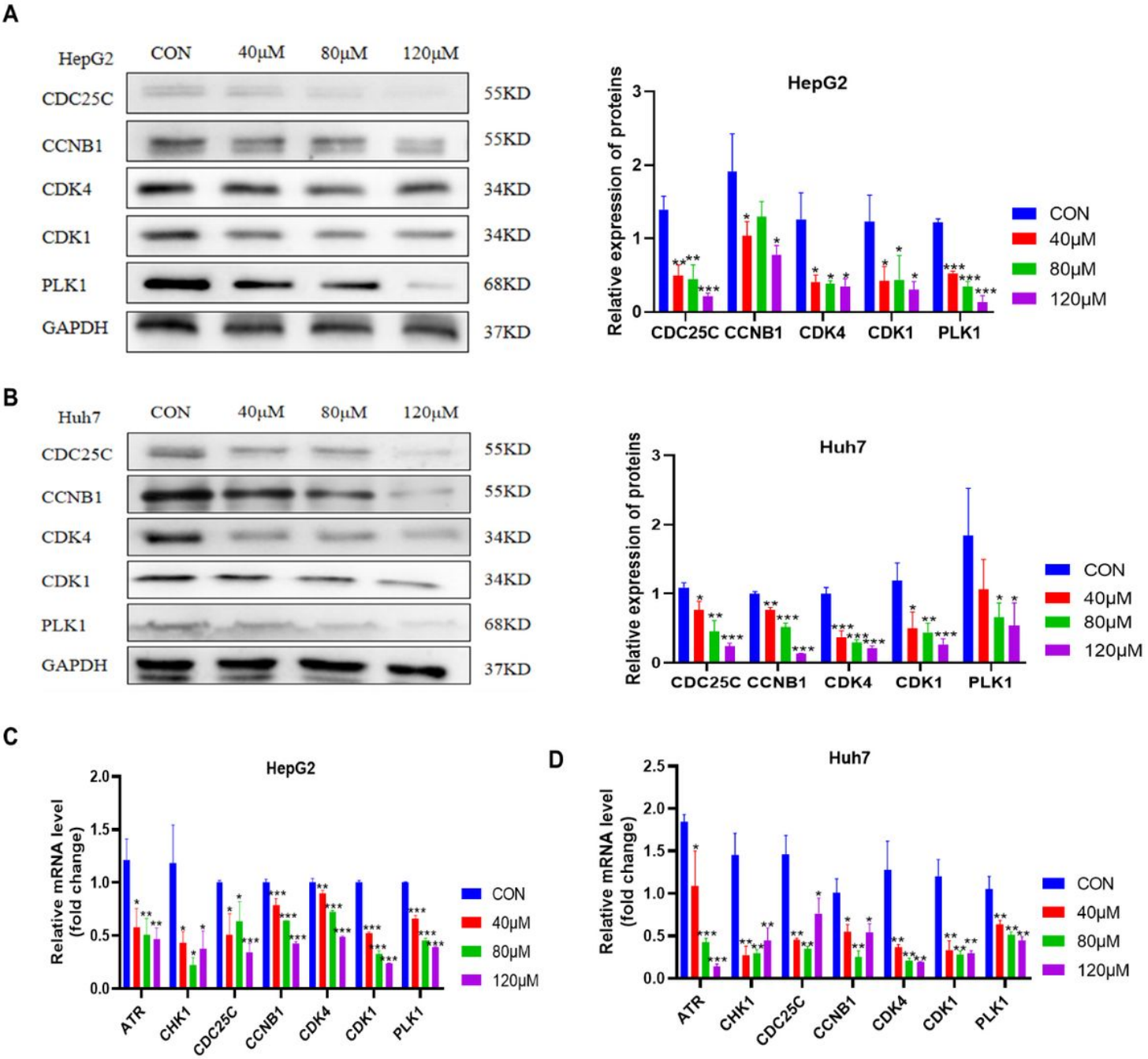


Figure 7

The effect of galangin on mRNA and protein expression of core targets in HCC cells. Effect of galangin on the expression of CDC25C, CCNB1, CDK4, CDK1, and PLK1 proteins in **(A)** HepG2 and **(B)** Huh7 cells. Effect of galangin on the expression of ATR, CHK1, CDC25C, CCNB1, CDK4, CDK1, and PLK1 mRNA in **(C)** HepG2 and **(D)** Huh7 cells.

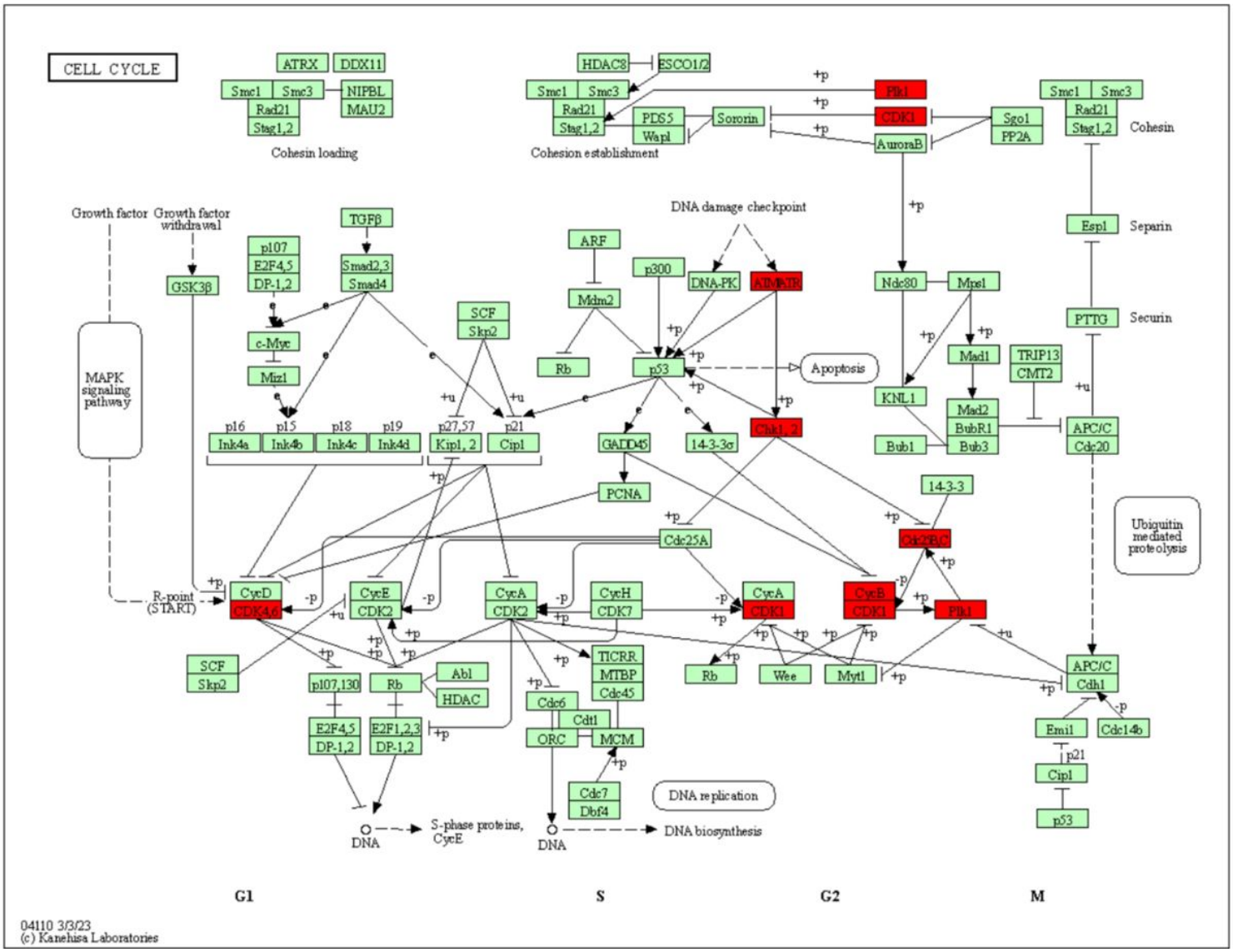


Figure 8

Cell cycle signaling pathway interfered by galangin