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ORIGINAL ARTICLE

Clinical and Translational Research

Targeting colorectal cancer with Herba Patriniae and Coix seed: Network pharmacology, molecular docking, and *in vitro* validation

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Abstract

BACKGROUND

Herba Patriniae and Coix seed (HC) constitute a widely utilized drug combination in the clinical management of colorectal cancer (CRC) that is known for its diuretic, anti-inflammatory, and swelling-reducing properties. Although its efficacy has been demonstrated in a clinical setting, the active compounds and their mechanisms of action in CRC treatment remain to be fully elucidated.

AIM

To identify the active, CRC-targeting components of HC and to elucidate the mechanisms of action involved.

METHODS

Active HC components were identified and screened using databases. Targets for each component were predicted. CRC-related targets were obtained from human

gene databases. Interaction targets between HC and CRC were identified. A "drug-ingredient-target" network was created to identify the core components and targets involved. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were conducted to elucidate the key pathways involved. Molecular docking between core targets and key components was executed. *In vitro* experiments validated core monomers.

RESULTS

Nineteen active components of HC were identified, with acacetin as the primary active compound. The predictive analysis identified 454 targets of the active compounds in HC. Intersection mapping with 2685 CRC-related targets yielded 171 intervention targets, including 30 core targets. GO and KEGG analyses indicated that HC may influence the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. Molecular docking showed that acacetin exhibited an optimal interaction with *AKT1*, identifying *PI3K*, *AKT*, and *P53* as key genes likely targeted by HC during CRC treatment. Acacetin inhibited HT-29 cell proliferation and migration, as well as promoted apoptosis, *in vitro*. Western blotting analysis revealed increased *p53* and cleaved caspase-3 expression and decreased levels of *p-PI3K*, *p-Akt*, and survivin, which likely contributed to CRC apoptosis.

CONCLUSION

Acacetin, the principal active compound in the HC pair, inhibited the proliferation and migration of HT-29 cells and promoted apoptosis through the PI3K/Akt/p53 signaling pathway.

Key Words: Colorectal cancer; Baijiangcao (Herba Patriniae); Yiyiren (Coix seed); Acacetin; Proliferation; Migration; Apoptosis; Network pharmacology

Core Tip: We identified acacetin as the key active ingredient of "Herba Patriniae and Coix seed." Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analyses suggested that "Herba Patriniae and Coix seed" acts on colorectal cancer (CRC) through the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, and molecular docking showed that the best docking effect was between acacetin and *AKT1*. *In vitro* experiment results corroborated that acacetin inhibits the proliferation and migration, and promotes apoptosis of CRC cells through the PI3K/Akt/p53 signaling pathway.

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INTRODUCTION

Colorectal cancer (CRC) is the third most frequently diagnosed cancer worldwide and accounts for approximately 10% of all cancer cases. Additionally, it is the second leading cause of cancer-related deaths worldwide, emphasizing its significant impact on global health[1]. The incidence of CRC is rising annually, with the age of onset decreasing, and mortality from recurrence and metastasis remaining a significant challenge[2]. The etiology of CRC is multifaceted and closely associated with various factors, such as age, sex, lifestyle, obesity, smoking, and dietary habits[3]. Most CRCs are sporadic and undergo slow progression from adenocarcinoma to carcinoma. Approximately 10% of adenomatous polyps develop into adenocarcinoma, which is proportional to the polyp size. In addition, CRC is closely associated with familial and hereditary syndromes. People with a family history of CRC have an increased risk of disease, including those with genetic syndromes (for example, lynch syndrome or familial adenomatous polyposis)[4]. Some studies have found that patients with a history of inflammatory bowel disease are prone to metastasis, with reduced histological differentiation, and increased lymphovascular and perineural invasion when compared with patients with sporadic CRC[5]. Surgery complemented by radiotherapy, chemotherapy, and targeted therapy is the primary treatment option for CRC. Traditional Chinese medicine (TCM) plays a pivotal role in the prevention and treatment of CRC, particularly during various stages of comprehensive treatment[6]. TCM significantly mitigates adverse reactions from radiotherapy and chemotherapy, enhances disease immunity, improves patient quality of life and survival rates, and reduces tumor recurrence and metastasis rates[7]. A randomized controlled study of the Chinese herbal compound, PRM1201[8], indicated that adjunctive chemotherapy could extend progression-free survival post-CRC surgery. In a previous study conducted by Yang Yufei's team[9], a prospective cohort of 222 patients who underwent radical treatment for CRC was followed over a period of five years. The findings revealed that patients treated with Sijunzi Decoction and additional TCMs exhibited substantially lower rates of cancer recurrence and metastasis than those who received only conventional therapy (21.38% *vs* 38.18%). Furthermore, the average duration before the onset of recurrence and metastasis in patients

receiving the combination of Sijunzi Decoction and TCMs was significantly longer (26.5 months) compared to that in the group receiving conventional treatment (16.0 months). Notably, the median time delay from recurrence to metastasis in the combination treatment group was 10.5 months, and this group reported no instances of liver metastasis. A retrospective cohort study[10] demonstrated that TCM treatment has a positive impact on extending the median overall survival in patients with CRC (75 months *vs* 65 months).

TCM asserts that the core pathological factors of CRC include dampness, heat, deficiency, and blood stasis, with most of the pathological properties characterized by a damp-heat turbidity-stasis interaction. Furthermore, damp-heat accumulation is common in the pathogenesis of CRC. The invasion and accumulation of dampness and heat in the colon disrupt the smooth flow of qi and blood, leading to obstruction by heat stasis toxins in the intestines, which ultimately results in cancer. Core issues include deficiency syndrome, particularly in the spleen and kidneys, and intestinal weakness. Empirical evidence has primarily identified dampness-heat stasis toxin as the main pathological factor, with clinical treatments focusing on heat-clearing, dampness-removing, anticancer, and blood-activating drugs. Herba Patriniae and Coix seed (HC) is a widely utilized drug combination in the clinical management of CRC, offering diuretic, pus-clearing, blood-dispersing, and anti-inflammatory effects. *Patrinia* and Coix seeds constitute the primary components of the classical formulation of Yiyi Fuzi Baijiang powder (YFBP), initially documented in the Synopsis of the Golden Chamber by the outstanding Chinese medicine scholar, Zhang Zhongjing (Eastern Han Dynasty, 150-219 AD). This formulation comprises three TCM ingredients (Coix seed, aconite seed, and Herba Patriniae) blended in a ratio of 30:6:15. In the TCM field, this formulation has been traditionally used to treat gastrointestinal (GI) disorders. Previous research demonstrated that the clinical application of YFBP may enhance the quality of life of patients with CRC, and preliminary studies have shown promising results for its use in CRC treatment. Moreover, another study identified that YFBP effectively inhibits tumor growth by disrupting the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway in CRC mouse models[11]. Further investigations demonstrated that YFBP significantly slowed CRC progression in $Apc^{Min/+}$ mice. This was achieved through modulation of the gut microbiota and a reduction in the production of regulatory T cells, offering a potential mechanism that explains its therapeutic effects[12]. The perennial herbs, *Patrinia scabiosifolia* Fisch and *P. illosa* Juss, were first documented in the Shennong Herbarium. These herbs are known for their ability to clear heat, detoxify, eliminate abscesses and pus, remove blood stasis, and relieve pain[13]. Flavonoids, triterpenoid saponins, iridoid terpenes, volatile oils, sterols, and phenylpropanoids (coumarins and lignans) are the primary active constituents extracted from *Patrinia*, with flavonoids and saponins identified as the key anti-tumor agents[14]. *Coix lachryma-jobi* has garnered significant attention owing to its wide range of biological activities. This herb, mainly planted in Asia and Africa, as well as the marginal areas of the Mediterranean, has the capacity to clear heat and dampness[15]. Coix seed comprises various bioactive components, including polysaccharides, proteins, polyphenols, oils, cinnamolactones, and sterols, among others. Notably, Coix seed oil (CSO) exhibits significant anticancer efficiency[16].

The bioactive components and mechanisms underlying the CRC inhibitory activity of HC remain unclear. Network pharmacology studies of TCM aim to explore the complex mechanisms of diseases, TCM syndromes, and herbal efficiency. It integrates systems biology, multi-pharmacology, and molecular networks. This approach provides a comprehensive description of the connections among the drug, related targets, and the disease, presented as a visual "active ingredient-target-disease" network[17]. Molecular docking is used to simulate the interactions between small molecules and proteins, and has seen considerable expansion in its application for investigating the mechanisms of action of new drugs in the last few years. Researchers have utilized a combination of network pharmacology, molecular docking, and experimental methods to investigate the active components, potential targets, and molecular mechanisms of HC in the treatment of CRC. These findings were preliminarily verified using cell-based experiments. The results indicated that acacetin is a key active component of HC, which exerts its anti-CRC function by suppressing tumor cell proliferation and promoting apoptosis *via* the PI3K/Akt/p53 signaling pathway. This study aimed to provide a series of references and an innovative foundation for the clinical applications and fundamental research on HC in CRC treatment.

MATERIALS AND METHODS

Network pharmacology analysis

Identification of active compounds and forecasting of putative targets: Using "patrinia" and "Coiren" as keywords, data were searched for on the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP; https://old.tcmsp-e.com/tcmsp.php). Retrieval criteria were set as oral bioavailability ≥ 30% and drug-likeness (DL) ≥ 0.18[18]. Further screening was conducted *via* the Swiss ADME platform (http://www.swissadme.ch), selecting compounds with "high" GI absorption and DL affirmed by at least two "Yes" responses. This study screened for viable components in *Patrinia* and Coix seed. Active components were identified by retrieving their SMILES notation *via* PubChem (https://pubchem.ncbi.nlm.nih.gov) based on their unique identifiers. Chemical targets were predicted by integrating the data with the Swiss Target Prediction database (http://www.swisstargetprediction.ch). Target and duplicate data with a confidence value of 0 were excluded.

Forecasting CRC-associated target genes: Using "Colorectal Cancer" as a keyword, CRC targets were identified in the GeneCards (https://www.genecards.org) and OMIM (https://www.omim.org) databases, and further explored in the DisGeNET database (https://www.disgenet.org). The top 2000 targets were selected through median screening in Microsoft Excel. The genes identified from the three databases were aggregated, and the duplicates were removed[19].

Construction of the HC-CRC protein-protein interaction network: Using Venny 2.1.0 (http://www.liuxiaoyuyuan.cn), intersection targets between CRC and HC were identified and imported into the STRING database (https://

cn.string-db.org) to obtain protein-protein interaction (PPI) networks. The PPI network was analyzed using Cytoscape 3.9.1, with thresholds set for Closeness (> 0.003), Betweenness (> 149.94), and Degree (> 34.15) to screen for core targets [20].

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses: To identify intersecting targets, the DAVID database (https://david.ncifcrf.gov) was utilized for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses. The filter condition was set with "Homo sapiens" as the species, and a significance threshold of $P < 0.05$ was established. GO enrichment analysis yielded results for biological process (BP), cellular component (CC), and molecular function (MF). This included an analysis of the KEGG signaling pathways. The selection criteria focused on the smallest P values, prioritizing the top 10 GO terms and top 20 KEGG pathways for further analysis. These were then imported into a bioinformatics platform (http://www.bioinformatics.com.cn) for visual analysis[21].

Integration of drug, component, target gene, pathway, and disease interaction networks: Data comprising *Patrinia*-Coix seed active ingredients, HC-CRC intersection targets, pathways, and diseases were input into Cytoscape 3.9.1 for 3D network visualization. In this network, the active ingredients and targets were designated as nodes, whereas the edges represented the interactions between these nodes[22].

Molecular docking: Molecular docking was performed for the five target proteins with the highest Degree scores according to the PPI network, which corresponded to the five active ingredients with the most intersection targets. The 3D structures of the compounds and target proteins were downloaded from PubChem and processed using AutoDock Tools 1.5.6 for the removal of crystal water, addition of hydrogen atoms, identification of rotatable bonds, structural optimization, and energy minimization. The structures were saved in pdbqt format. Binding energy calculations were performed using AutoDock Vina 1.1.2, with docking results visualized using PyMOL 2.1, and LigPlot + 2.2.8 used for analyzing the interaction modes between compounds and target proteins. This analysis included the specifics of compound-protein residue interactions and hydrogen bonding effects. Subsequently, the binding affinities of the screened compounds for their targets were estimated based on their binding energies[23,24].

Experimental verification

Drugs and reagents: A range of reagents and supplies were utilized in this study, including acacetin (lot number: 00017; Sigma-Aldrich, St. Louis, MO, United States) and RPMI-1640 medium (lot number: 10491; Solarbio, Beijing, China). Extra-grade fetal bovine serum was sourced from the Wuhan Puroxai Company (lot number: 164210; Wuhan, China), and antibiotics (penicillin-streptomycin double antibody) obtained from Gibco (lot number: 15140-122; Waltham, MA, United States). Cell culture and apoptosis detection kits, such as the Trypsin-EDTA (0.25%, lot number: 25200056; Gibco) and Annexin V-FITC Apoptosis Detection Kits (lot number: 556547; BD Biosciences, Franklin Lakes, NJ, United States), were used. Additionally, the key proteins, *PI3K*, *AKT*, and *P53*, as well as antibodies against them, were purchased from Abcam (with respective lot numbers: Ab151549, ab179463, and ab131442; Cambridge, United Kindom). Phosphorylation status was assessed using phospho-*PI3* kinase p85/p55 (batch No. 4228; Cell Signaling Technology, Danvers, MA, United States) and phospho-*Akt* (Ser473) XP rabbit monoclonal (lot number: 4060; Cell Signaling Technology) antibodies. Apoptotic markers, including cleaved caspase-3 (lot number: 9661; Cell Signaling Technology) and survivin (lot number: 2808; Cell Signaling Technology), along with the secondary antibodies, anti-rabbit IgG (lot number: 7074P2; Cell Signaling Technology) and β-actin (lot number: AF7018; Affinity Biosciences, Zhenjiang, China), were also utilized in the analysis (Figure 1).

Experimental instruments: In this study, a variety of specialized laboratory equipment was utilized to ensure the precision of the experimental results. This included an optical inverted microscope from Nikon (model ECLIPSE Ts2R; Tokyo, Japan) for cell observation and a low-speed centrifuge from the Baiyang Centrifuge Factory (model B600-A) for sample preparation. Our laboratory also employed a Super Clean Table from the Beijing Changping Great Wall Air Purification Engineering Company (Beijing, China) for maintaining a contaminant-free environment, alongside a 37 °C and 5% CO_2 Constant Temperature Aseptic Incubator from Sanyo (model MCO-18AIC; Osaka, Japan) for cell culture. Cells were counted on Marienfeld plates (lot number: 0650030; Paul Marienfeld, Germany). Cell populations were analyzed using a flow cytometer (BD FACSCanto; BD Biosciences), and enzyme activity was measured using an enzyme marker from BioTek (Synergy HT; Winooski, VT, United States). Additionally, for sample centrifugation, a low-temperature and high-speed centrifuge from Sigma-Aldrich (model 3-18K/3K30) was used. Protein analysis was performed using a suite of equipment from Bio-Rad (Hercules, CA, United States), including a protein electrophoresis device, protein electrophoresis transfer device, and gel imaging system (GelDoc XR Bio-Rad).

Cell culture: HT-29 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cultures were maintained in a 5% CO_2 incubator at 37 °C. Upon reaching 80%-90% confluence, the cells were digested with 0.25% EDTA. Only cells in the growth phase were selected for subsequent experiments.

Cell viability assay: HT-29 cells were seeded into 96-well plates at a density of 5×10^4 cells/well. After 24 hours of incubation, they were treated with various concentrations of acacetin (440.000, 220.000, 110.000, 55.000, 27.500, 13.750, 6.875, and 0 μM). Additionally, blank (containing complete culture medium without cells) and control wells (containing cells in complete culture medium but without the drug) were established. After 24 and 48 hours of incubation, the medium was replaced with fresh medium containing a 10% cell counting kit-8 (CCK-8) solution, and the plates were

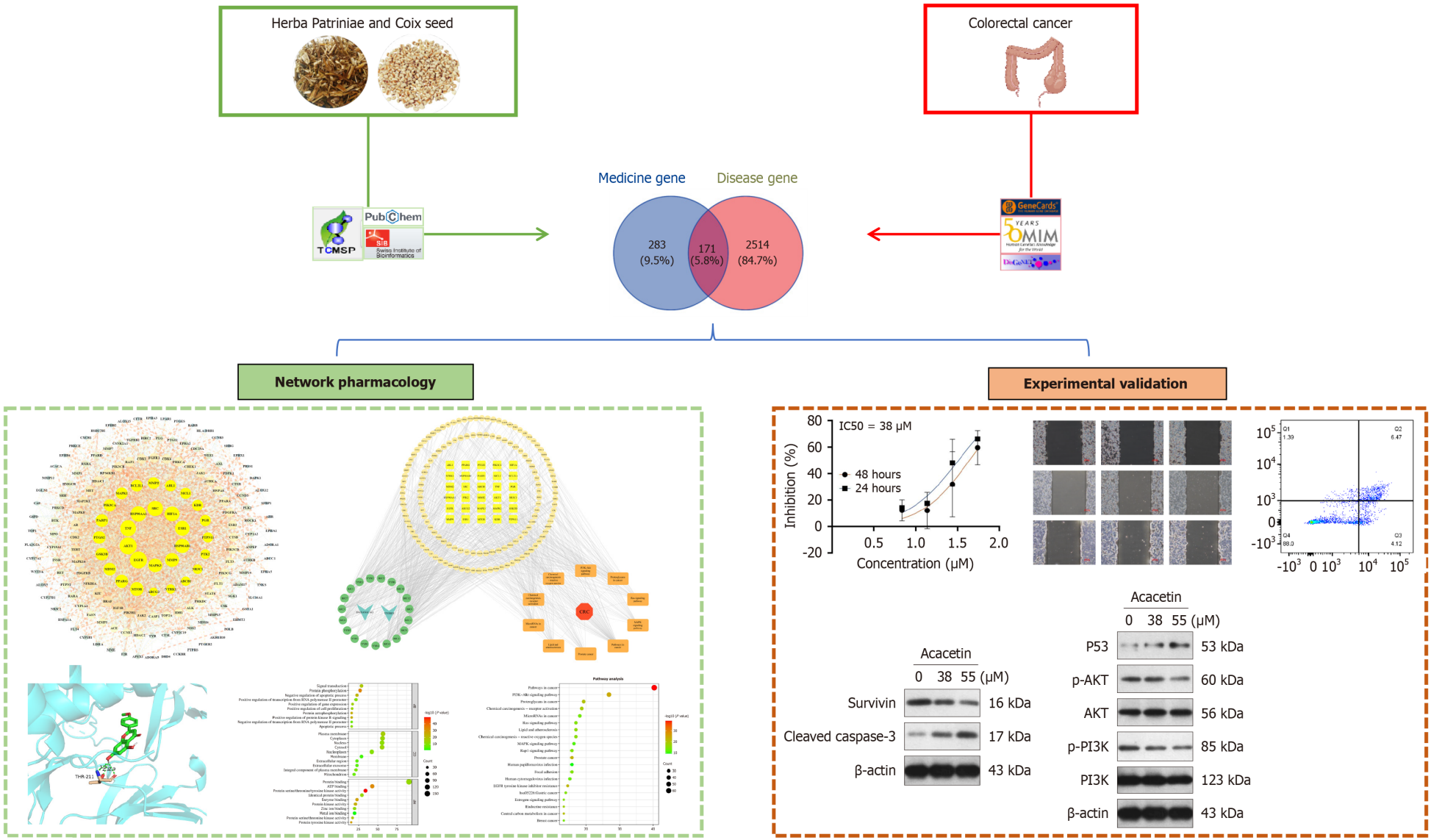


Figure 1 Flow chart depicting the study methodology. Various databases were searched to obtain common targets of colorectal cancer (CRC) and Herba Patriniae-Coix seed. Possible targets and pathways for CRC were analyzed *via* network pharmacology and finally verified through *in vitro* experiments.

subsequently incubated for an additional 1 hour in the dark. The absorbance of each well was measured at 450 nm using an enzyme-linked immunosorbent assay reader. The cell survival rate was calculated using the following formula: Cell survival rate (%) = (Experimental group absorbance - Blank group absorbance)/(Control group absorbance - Blank group absorbance) × 100.

Based on the results, the optimal concentration of acacetin was determined for further experiments.

Wound healing assay: The migration of HT-29 cells was measured using a cell scratch assay. Cells were seeded in 6-well plates at a density of 1 × 10^6 cells/well and cultured for 24 hours. Subsequently, a vertical scratch was made along the center of each well. The wells were then treated with various concentrations of acacetin. Photographs were taken at 0 hour, 24 hours, and 48 hours after wound-scratching. The scratch distance was quantified using ImageJ software, and the cell migration rate was assessed using the following formula: Cell migration rate (%) = (Initial distance - final distance)/initial distance × 100.

Flow cytometry assay: HT-29 cells were cultured in 6-well plates at a density of 1 × 10^6 cells/well and incubated for 24 hours. Following treatment with various concentrations of acacetin for 24 hours, the cells were harvested and resuspended in 1 × Binding Buffer. To each tube, 5 μL Annexin V-FITC and PI antibody were added, along with 100 μL of the cell suspension. The mixture was then mixed gently. After incubation in a dark room at an appropriate temperature for 15 minutes, 400 μL of 1 × Binding Buffer was added to the cells, which were then measured *via* flow cytometry for up to 1 hour.

Western blotting analysis: After treatment with varying concentrations of acacetin for 24 hours, the cells were harvested, lysed, and centrifuged to extract total protein. The protein concentration was quantified using a BCA protein detection kit. Proteins were denatured at 100 °C for 8 minutes. A 20 μg protein sample was subjected to SDS-PAGE at 140 V for 1 hour, followed by 100 V post-electrophoresis. The samples were then cooled on ice for 1 hour before being transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skim milk powder for 2 hours, followed by the addition of the primary antibody and overnight incubation at 4 °C. Subsequently, the primary antibody was removed, and the membrane was washed three times with 1 × PBST solution (5 minutes per wash). The secondary antibody was then incorporated and incubated at room temperature for 1 h, followed by removal and three washes with 1 × PBST solution. A 1:1 mixture of chemiluminescence kit A-B was applied to the PVDF membrane and incubated for 30 seconds. Target protein bands were imaged using a gel imaging system, and the grayscale values of each band were analyzed using Image Lab software. The grayscale ratios of cleaved caspase-3, survivin, *PI3K*, *p-PI3K*, *Akt*, *p-Akt*, *P53*, and β-actin were employed to quantify the relative protein expression levels in each group.

Statistical analysis

All data were analyzed using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY, United States), and GraphPad Prism, version 10.0 (GraphPad Software, La Jolla, CA, United States), and expressed as the mean ± SD. One-way analysis of variance was used to assess differences between groups, with $P \leq 0.05$ set to determine statistically significant differences.

RESULTS

Active compounds and putative targets of HC

Fifty-two active ingredients and 38 Coix seed variants were identified using the TCMSP platform. Utilizing the screening criteria of an oral bioavailability ≥ 30%, DL ≥ 0.18, "high" GI absorption as per the Swiss ADME database, and at least two "Yes" responses for DL, 12 active ingredients were identified in *Patrinia* and seven in Coix seed. Nineteen active ingredients were ultimately screened, of which sitosterol (MOL000359) and stigmasterol (MOL000449) were common to both herbs. Canonical SMILES for these 19 compounds were retrieved from the PubChem database and input into the Swiss Target Prediction database. After eliminating targets with a confidence value of 0 and duplicate entries, 454 potential targets of the HC active ingredients were identified. The five compounds with the highest number of targets were acacetin, luteolin, quercetin, bolusanthol B, and sinoacutine. Table 1 shows information related to the active ingredients identified in HC.

Potential CRC and HC intersection targets

From the GeneCards, DisGeNET, and OMIM databases, 1600, 1206, and 500 CRC targets were identified, respectively. By merging these lists and eliminating duplicate entries, a total of 2685 CRC-related targets were compiled. A Venn diagram (Figure 2) was used to illustrate the identification of 171 intersecting targets between HC and CRC.

PPI network and hub targets analyses

To elucidate the relationships among the 171 intersecting target molecules, a PPI network was constructed using the STRING database and further visualized *via* Cytoscape 3.9.1 software (Figure 3), which consisted of 171 nodes and 2920 edges (Figure 3A). Following the 2.1.3 screening criteria, 30 nodes and 369 edges were identified (Figure 3B and C). The CentiScaPe 2.2 plugin was used to analyze the target set and isolate the core targets. Table 2 shows the top 10 core genes identified. Furthermore, alluvial diagrams were used to illustrate the relationships among the two herbs, eight core components, and nine core genes (Figure 3D). Node connectivity is depicted through variations in the sizes of the nodes

Table 1 Basic information of the 19 active ingredients in Herba Patriniae-Coix seed

Herb	Mol ID	Molecule name	Oral bioavailability (%)	Drug-likeness	Target amount
BJC1	MOL001676	Vilmorrianine C	33.96	0.22	59
BJC2	MOL001677	Asperglaucide	58.02	0.52	100
BJC3	MOL001678	Bolusanthol B	39.94	0.41	100
BJC4	MOL001689	Acacetin	34.97	0.24	100
BJC5	MOL002322	Isovitexin	31.29	0.72	3
BJC6	MOL001697	Sinoacutine	63.39	0.53	100
BJC7	MOL000358	Beta-sitosterol	36.91	0.75	44
BJC8	MOL000359	Sitosterol	36.91	0.75	44
BJC9	MOL000422	Kaempferol	41.88	0.24	3
BJC10	MOL000449	Stigmasterol	43.83	0.76	41
BJC11	MOL000006	Luteolin	36.16	0.25	100
BJC12	MOL000098	Quercetin	46.43	0.28	100
YYR1	MOL001323	Sitosterol alpha 1	43.28	0.78	60
YYR2	MOL001494	Mandenol	42.00	0.19	100
YYR3	MOL002882	[(2R)-2,3-dihydroxypropyl](Z)-octadec-9-enoate	34.13	0.30	41
YYR4	MOL000359	Sitosterol	36.91	0.75	44
YYR5	MOL000449	Stigmasterol	43.83	0.76	41
YYR6	MOL008121	2-Monoolein	34.23	0.29	52
YYR7	MOL000953	CLR	37.87	0.68	46

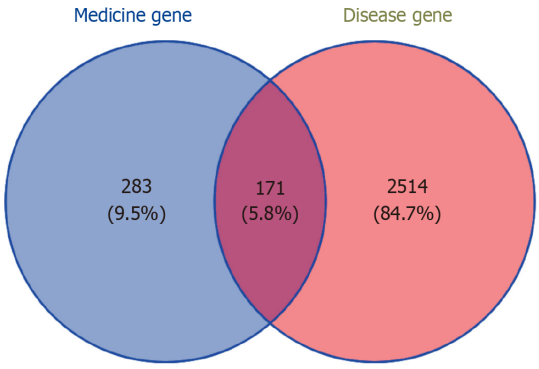
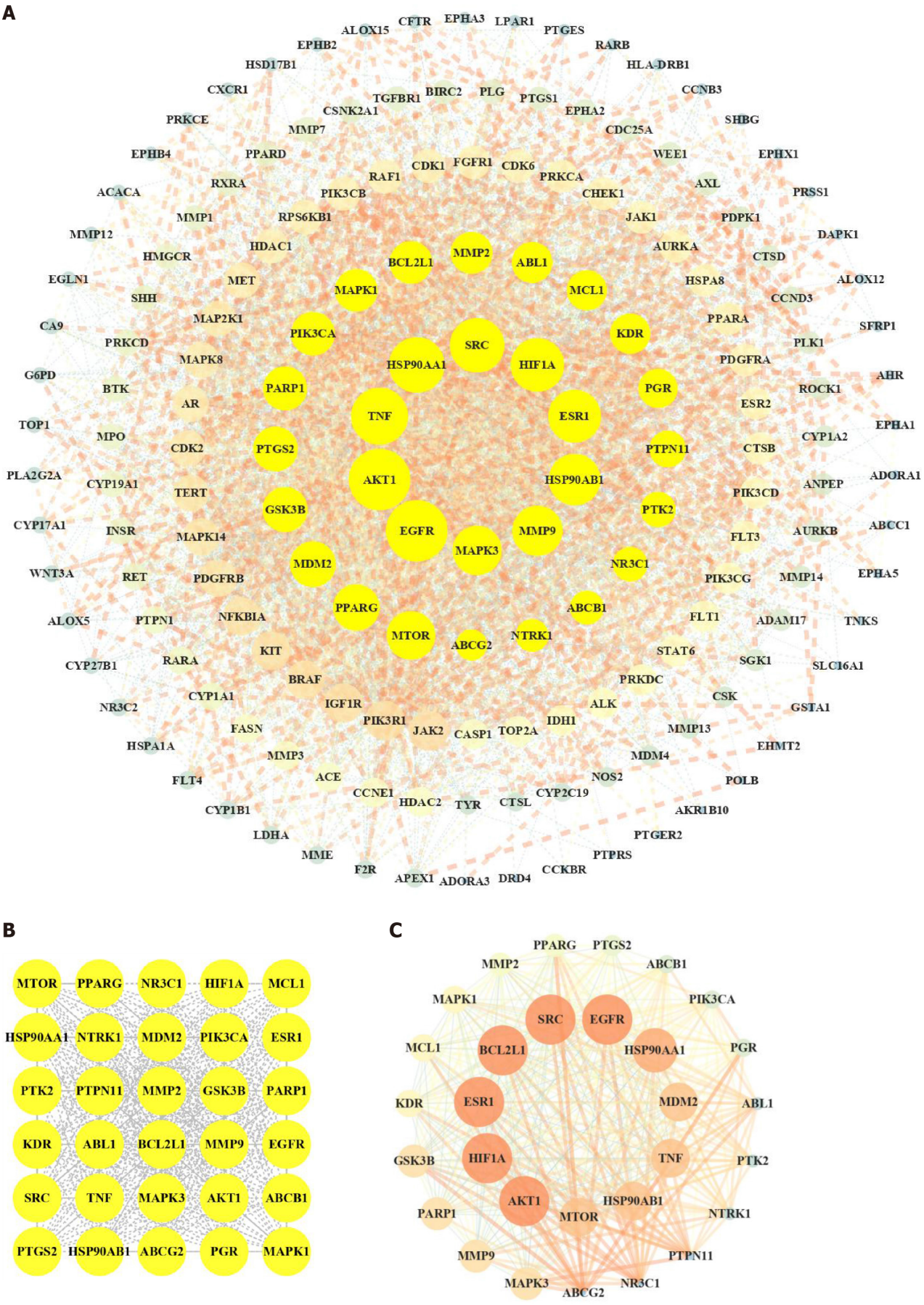


Figure 2 Venn diagram of the active ingredients and disease targets.

and edges, as well as through color intensity. Larger sizes and darker colors indicate stronger connections within the network.

GO and KEGG pathway enrichment analyses

The key biological functions of HC for CRC treatment were elucidated through GO and KEGG enrichment analyses. GO analysis revealed that the numbers of CC, MF, and BP processes were 91, 149, and 614, respectively ($P < 0.05$). Based on prior results, the top five CC processes comprised the plasma membrane, cytoplasm, cytosol, nucleus, and nucleoplasm. The top five MF processes included protein binding, ATP binding, protein serine/threonine/tyrosine kinase activity, identical protein binding, and enzyme binding. The first five BPs identified were signal transduction, protein phosphorylation, negative regulation of the apoptotic process, transcription from the RNA polymerase II promoter, and positive regulation of gene expression. The top 10 bar and bubble charts are illustrated in Figure 4. After KEGG enrichment analysis, 154 signaling pathways were identified ($P < 0.05$). The top five signaling pathways identified included pathways in cancer, the *PI3K/Akt* signaling pathway, proteoglycans in cancer, chemical carcinogen-receptor activation, and microRNAs in cancer; HC can likely target CRC through these pathways. A bubble diagram of the first 20 bars is shown in Figure 5.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ALOX15
CFTR
EPHA3
LPAR1
PTGES
EPHB2
RARB
HSD17B1
HLA-DRB1
CXCR1
TGFBR1
BIRC2
PLG
PTGS1
CCNB3
CSNK2A1
EPHA2
PRKCE
MMP7
CDC25A
SHBG
EPHB4
PPARD
CDK1
FGFR1
CDK6
WEE1
EPHX1
RAF1
PRKCA
RXRA
AXL
ACACA
PIK3CB
CHEK1
PRSS1
MMP1
RPS6KB1
JAK1
PDPK1
MMP12
DAPK1
HDAC1
AURKA
HMGCR
BCL2L1
MMP2
ABL1
CTSD
EGLN1
MET
MAPK1
MCL1
HSPA8
ALOX12
SHH
CCND3
MAP2K1
PPARA
CA9
PIK3CA
SRC
KDR
SFRP1
PRKCD
PLK1
MAPK8
HSP90AA1
HIF1A
PDGFRA
G6PD
AHR
BTK
PARP1
PGR
ROCK1
AR
ESR2
TOP1
TNF
ESR1
EPHA1
MPO
CYP1A2
CDK2
PTGS2
PTPN11
CTSB
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AKT1
HSP90AB1
ADORA1
CYP19A1
TERT
ANPEP
PIK3CD
GSK3B
PTK2
CYP17A1
INSR
EGFR
MMP9
AURKB
ABCC1
MAPK14
MAPK3
FLT3
MDM2
NR3C1
WNT3A
RET
PDGFRB
PIK3CG
MMP14
EPHA5
PPARG
ABCB1
ALOX5
PTPN1
NFKBIA
FLT1
ADAM17
TNKS
MTOR
NTRK1
ABCG2
KIT
STAT6
CYP27B1
RARA
SGK1
SLC16A1
BRAF
PRKDC
CYP1A1
CSK
NR3C2
IGF1R
ALK
GSTA1
PIK3R1
IDH1
FASN
JAK2
CASP1
TOP2A
MMP13
HSPA1A
EHMT2
MMP3
MDM4
FLT4
ACE
NOS2
POLB
CCNE1
CYP2C19
HDAC2
TYR
CTSL
CYP1B1
AKR1B10
LDHA
PTGER2
MME
PTPRS
F2R
CCKBR
APEX1
ADORA3
DRD4
B
MTOR
PPARG
NR3C1
HIF1A
MCL1
HSP90AA1
NTRK1
MDM2
PIK3CA
ESR1
PTK2
PTPN11
MMP2
GSK3B
PARP1
KDR
ABL1
BCL2L1
MMP9
EGFR
SRC
TNF
MAPK3
AKT1
ABCB1
PTGS2
HSP90AB1
ABCG2
PGR
MAPK1
C
PPARG
PTGS2
MMP2
ABCB1
MAPK1
PIK3CA
SRC
EGFR
MCL1
BCL2L1
HSP90AA1
PGR
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MDM2
ABL1
GSK3B
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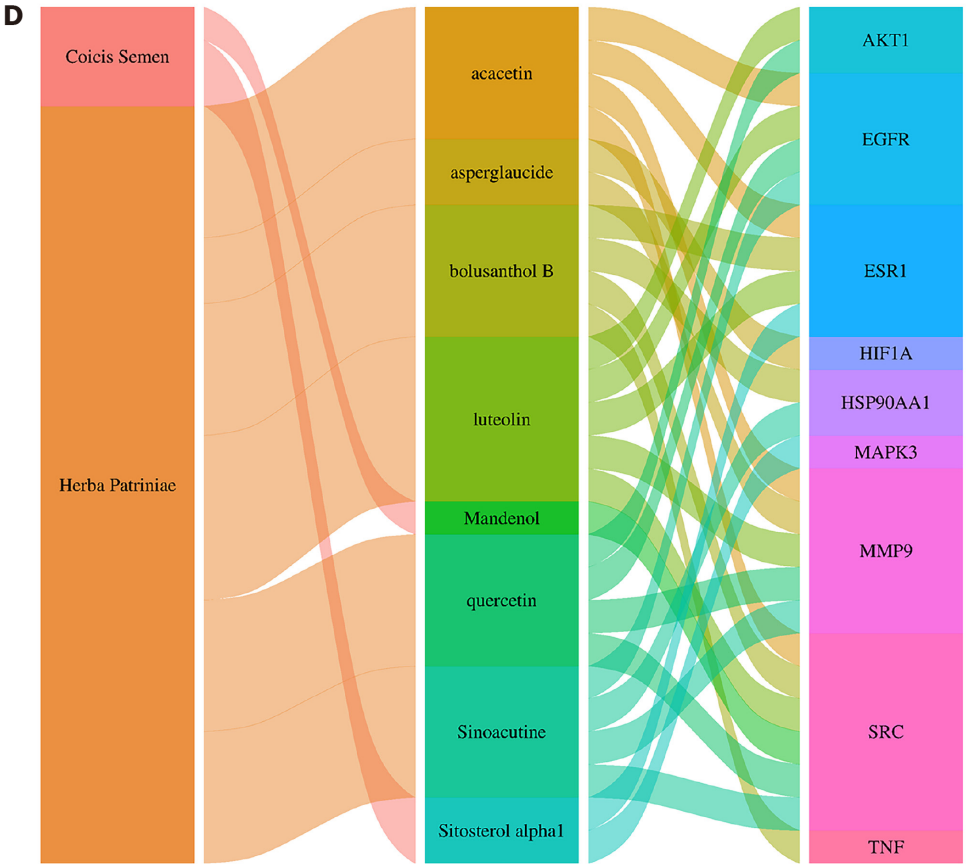

Figure 3 Protein-protein interaction analysis of the intersection targets between Herba Patriniae-Coix seed and colorectal cancer. A: The protein-protein interaction (PPI) network between the Herba Patriniae-Coix seed and colorectal cancer intersection targets; B and C: The top 30 core targets identified through PPI network analysis; D: The impact map revealing the relationship between traditional Chinese medicine, core components, and core genes.

Construction of an herbal composition-target-pathway-disease network

Two herbs, 19 active ingredients, 171 cross targets, the top 10 pathways, and CRC diseases were integrated into Cytoscape 3.9.1 to establish an herbal ingredient-target-pathway-disease network (Figure 6). Upon examining the network, the network diagram was found to comprise 203 nodes and 893 edges. Acacetin exhibited the highest value, followed by luteolin, quercetin, bolusanthol B, and sinoacutine. The signaling pathway that exhibited the highest degree of involvement was the "PI3K-Akt signaling pathway." Within a network, the degree value of a node depends on the number of edges connected to other nodes. Nodes with high degree values are often the key components of a network and play significant roles within it. Data pertaining to the major TCM components, signaling pathways, and targets are summarized in Tables 3, 4 and 5.

Molecular docking study

Five key target proteins (EGFR, AKT1, TNF, HSP90AA1, and SRC) were molecularly docked with five core components (acacetin, luteolin, quercetin, bolusanthol B, and sinoacutine), and the binding capacities of target and ligand proteins evaluated. The AutoDock software predicted the binding capacities, with all binding energies being < -6.0 kcal/mol (Figure 7). All five active ingredients demonstrated strong binding affinities for EGFR, AKT1, TNF, HSP90AA1, and SRC. Among these, acacetin, bolusanthol B, luteolin, and quercetin exhibited the most effective docking with *AKT1*. Consequently, molecular docking with the human target protein, *AKT1*, was conducted using AutoDock Vina 1.1.2, and the results are presented in Table 6. The findings indicated that the four compounds exhibited a significant binding effect with *AKT1*, with binding energies < -6.0 kcal/mol. The complex resulting from the compound-protein docking was synthesized using PyMOL 2.1 software, from which the binding mode was derived. The LigPlot + 2.2.8 software elucidated the amino acid residues of the compound and the protein pocket, as detailed in Figure 8. For example, the amino acid residues of luteolin and quercetin that interacted with *AKT1* included THR-211, TYR-272, and GLN-79. The interaction of acacetin and bolusanthol B with *AKT1* involved amino acid residues THR-211, ASP-274, and THR-81, demonstrating a well-matched molecular and protein interface.

Acacetin inhibits the proliferation of HT-29 cells

In this study, we examined the cytotoxicity and inhibitory effects of acacetin on HT-29 cells. Cell viability was measured using the CCK-8 assay after treatment with various concentrations of acacetin for 24 and 48 hours. Acacetin markedly inhibited HT-29 cell proliferation (Figure 9).

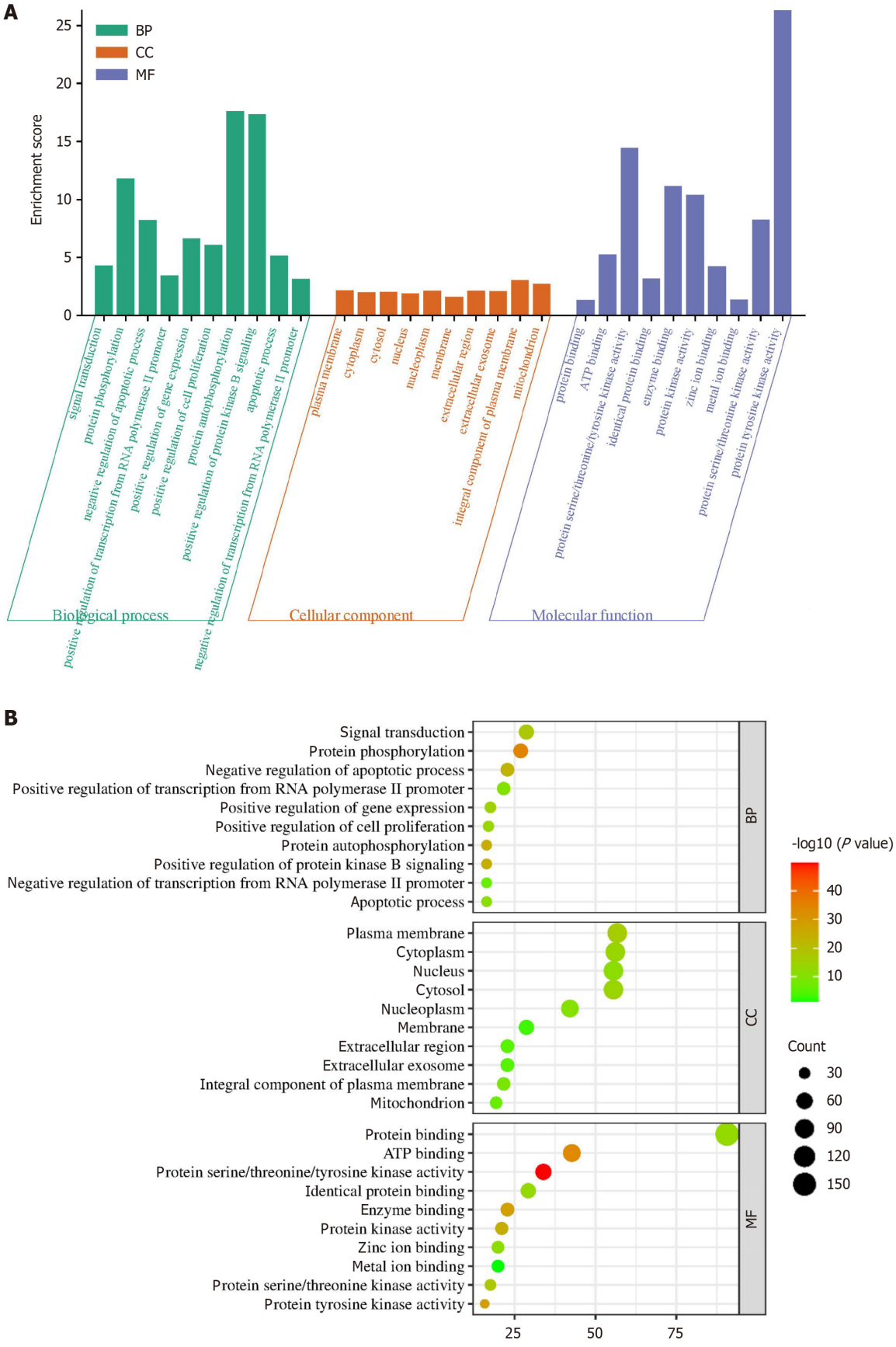

Figure 4 Gene ontology. A and B: Gene ontology functional enrichment analysis. BP: Biological process; CC: Cell component; MF: Molecular function.

Table 2 Top 10 core targets in the protein-protein interaction network between Herba Patriniae-Coix seed and colorectal cancer

Rank	Target	Degree
1	EGFR	122
2	AKT1	122
3	TNF	109
4	HSP90AA1	105
5	SRC	102
6	HIF1A	99
7	ESR1	97
8	HSP90AB1	94
9	MMP9	90
10	MAPK3	87

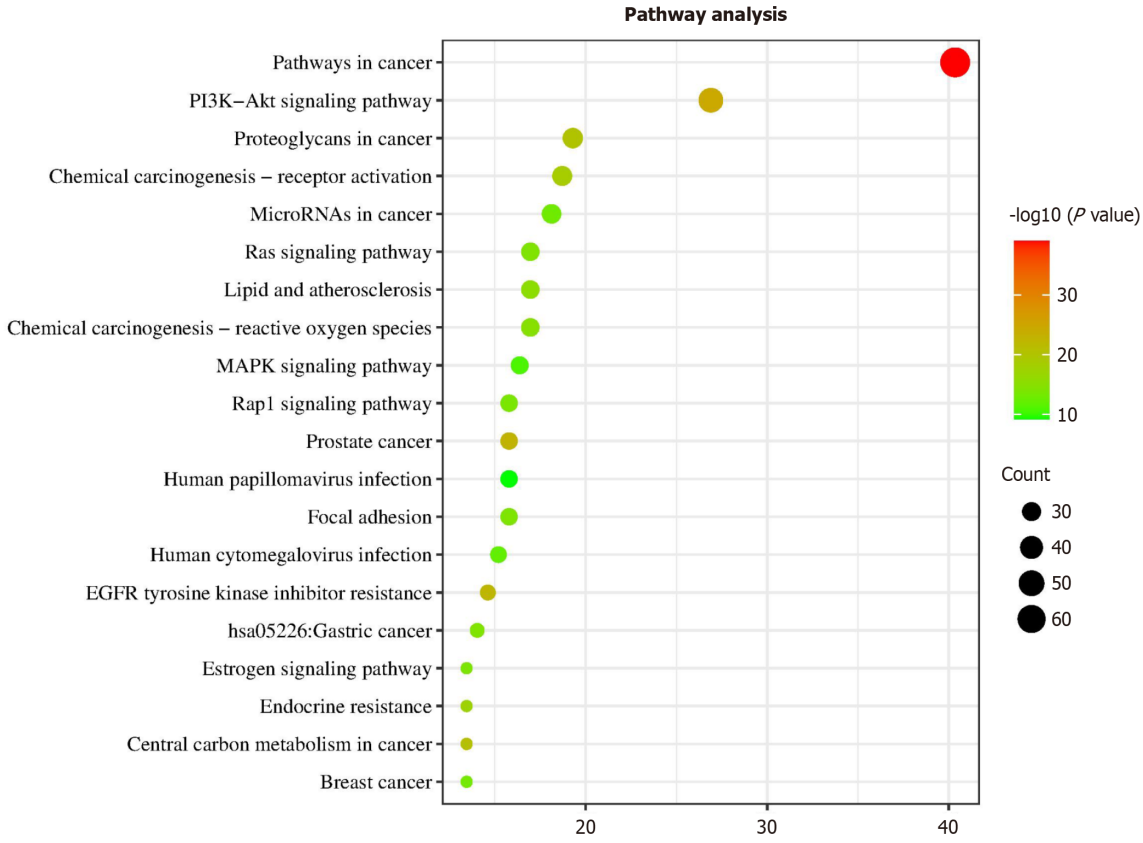

Figure 5 Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis. The X-axis denotes the enrichment rate of these genes within the total gene pool, whereas the Y-axis indicates the enrichment pathway of the target gene ($P \leq 0.05$). The depth of the color corresponds to the magnitude of the value, whereas the size of the circle reflects the enrichment count of these pathways. The top five signaling pathways are: Pathways in cancer, phosphoinositide 3-kinase-Akt signaling pathway, proteoglycans in cancer, chemical carcinogenesis, receptor activation, and microRNAs in cancer.

Acacetin inhibits HT-29 cell migration

Scratch assays were conducted on HT-29 cells that were treated with acacetin at concentrations of 0, 38, and 55 μM. As shown in Figure 10, HT-29 cells in the experimental group exhibited negligible migration compared with those in the control group ($P < 0.05$). Thus, acacetin significantly inhibited HT-29 cell migration.

Acacetin induced the apoptosis of HT-29 cells

The effect of acacetin on HT-29 cell apoptosis was further explored using flow cytometry. The results, as depicted in Figure 11, demonstrate that the apoptosis rate was significantly different from that in the control group ($P < 0.01$); acacetin significantly induced apoptosis in HT-29 cells. Expression levels of apoptosis-related proteins, including cleaved

Table 3 Top five active ingredients in the herbal ingredient-target-pathway-disease network

Molecule name	Degree	CC	BC
Acacetin	56	0.433	0.107
Luteolin	54	0.430	0.089
Quercetin	53	0.428	0.090
Bolusanthol B	49	0.419	0.148
Sinoacutine	45	0.414	0.127

CC: Closeness centrality; BC: Betweenness centrality.

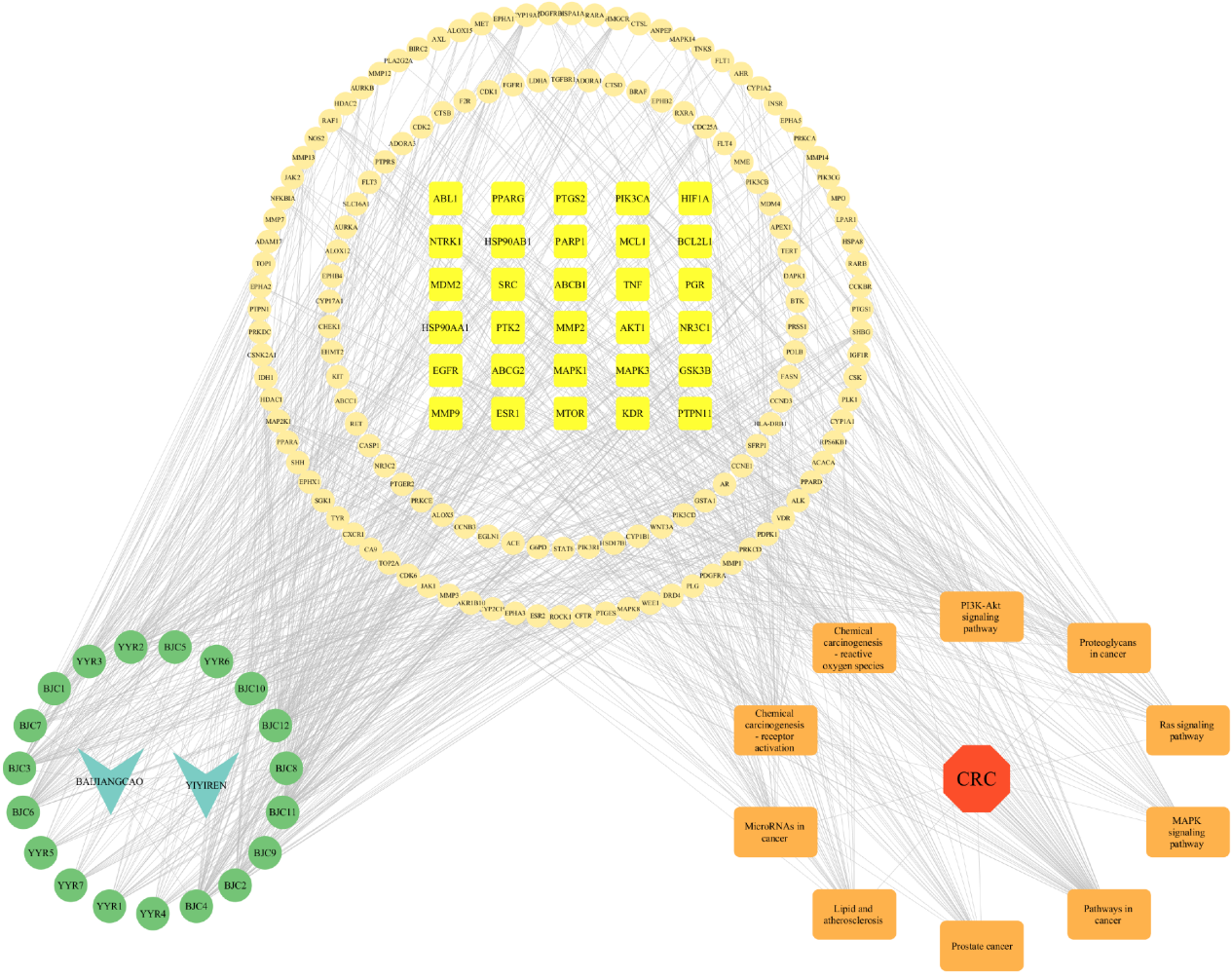

Figure 6 Herbal components-target-pathway-disease network for Herba Patriniae-Coix seed anti-colorectal cancer therapy. The light-green inverted triangles symbolize herbs, the dark-green circles signify the active ingredients of various herbs, the yellow circles denote general targets, the yellow squares indicate core targets, the orange rectangles correspond to pathways, and the red octagons represent diseases.

caspase-3 and survivin, were quantified *via* western blotting, which revealed that relative to the control group, expression levels of both proteins significantly increased following treatment with acacetin (38 μM and 55 μM; $P < 0.05$).

Influence of acacetin on the expression of proteins related to the PI3K/Akt/P53 signaling pathway

Network pharmacology analysis showed that the mechanism of action of acacetin in CRC likely involves tumor-related signaling pathways, including the PI3K/Akt pathway. P53, as a vital protein in cell cycle arrest and apoptosis, is a downstream target of the PI3K/Akt signaling pathway[25]. Consequently, regulation of the PI3K/Akt signaling pathway by acacetin was investigated. After 48 hours of acacetin treatment in HT-29 cells, the expression levels of *p-PI3K* and *p-Akt* were significantly reduced, whereas a significant increase in p53 protein expression was observed in the experimental group compared to those in the control group ($P < 0.05$) (Figure 12). This indicates that acacetin may regulate the prolif-

Table 4 Top five Kyoto Encyclopedia of Genes and Genomes pathways in the herbal ingredient-target-pathway-disease network

KEGG pathway name	Degree	CC	BC
Pathways in cancer	70	0.457	0.163
PI3K-Akt signaling pathway	47	0.404	0.052
Proteoglycans in cancer	34	0.393	0.030
Chemical carcinogenesis-receptor activation	33	0.391	0.038
MicroRNAs in cancer	32	0.388	0.035

CC: Closeness centrality; BC: Betweenness centrality; KEGG: Kyoto Encyclopedia of Genes and Genomes; PI3K: Phosphoinositide 3-kinase.

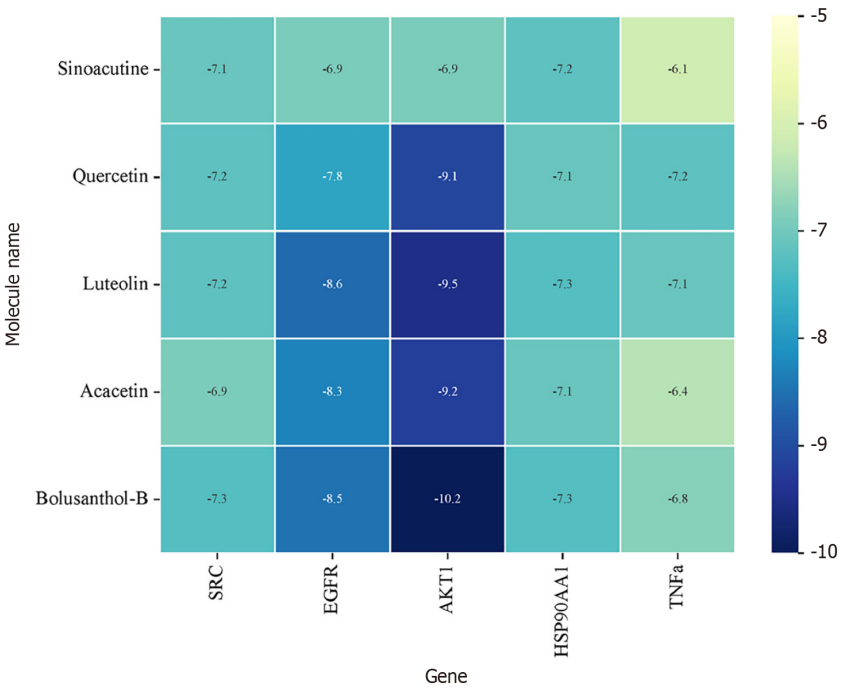

Figure 7 Heat map of molecular docking binding energy between the five core components and targets. All five active components showed a strong binding affinity for EGFR, AKT1, TNF, HSP90AA1, and SRC. Among them, acacetin, bolusanthol B, luteolin, and quercetin exhibited the best docking effect with AKT1.

eration and apoptosis of HT-29 cells *via* the PI3K/Akt/P53 signaling pathway, thereby corroborating the network pharmacology findings.

DISCUSSION

CRC is mainly divided into two types according to its location: Colon cancer, which accounts for 71% of CRC cases, and rectal cancer, which comprises 29% of cases[26]. In 2020, estimates indicated that there were over 1.9 million new cases of CRC globally, resulting in more than 930000 deaths. By 2040, the global incidence of CRC is projected to escalate to 3.2 million new cases annually, representing a 63% increase, with deaths expected to reach 1.6 million per year, representing a 73% increase. The incidence of CRC in adolescents, young adults, and adults is rising annually[27]. In European countries, approximately 11% of CRC cases are attributed to being overweight and obese, particularly due to visceral or abdominal fat. Epidemiological research has indicated that 30%-70% of obese men have an increased risk of CRC. The most prevalent CRC subgroups include the colon, proximal colon, distal colon, and rectum[28]. CRC pathogenesis is influenced by a variety of factors, including poor dietary habits, a sedentary lifestyle, smoking, excessive alcohol intake, obesity, alterations in the gut microbiota, diabetes, and genetic mutations[29,30]. Currently, surgery, radiotherapy, and chemotherapy are the primary treatments for CRC. For patients diagnosed with recurrent and metastatic late-stage CRC, treatment often involves repeated surgery, chemotherapy, radiation therapy, targeted therapy, or a combination thereof [31]. Despite diagnostic and therapeutic advances, the prognosis for patients with CRC remains poor. Furthermore, long-term therapy can result in serious side effects and toxicity, such as nausea, vomiting, mouth ulcers, diarrhea, hepatotoxicity, bone marrow suppression, and immunosuppression, severely limiting their clinical application. Thus, there is an

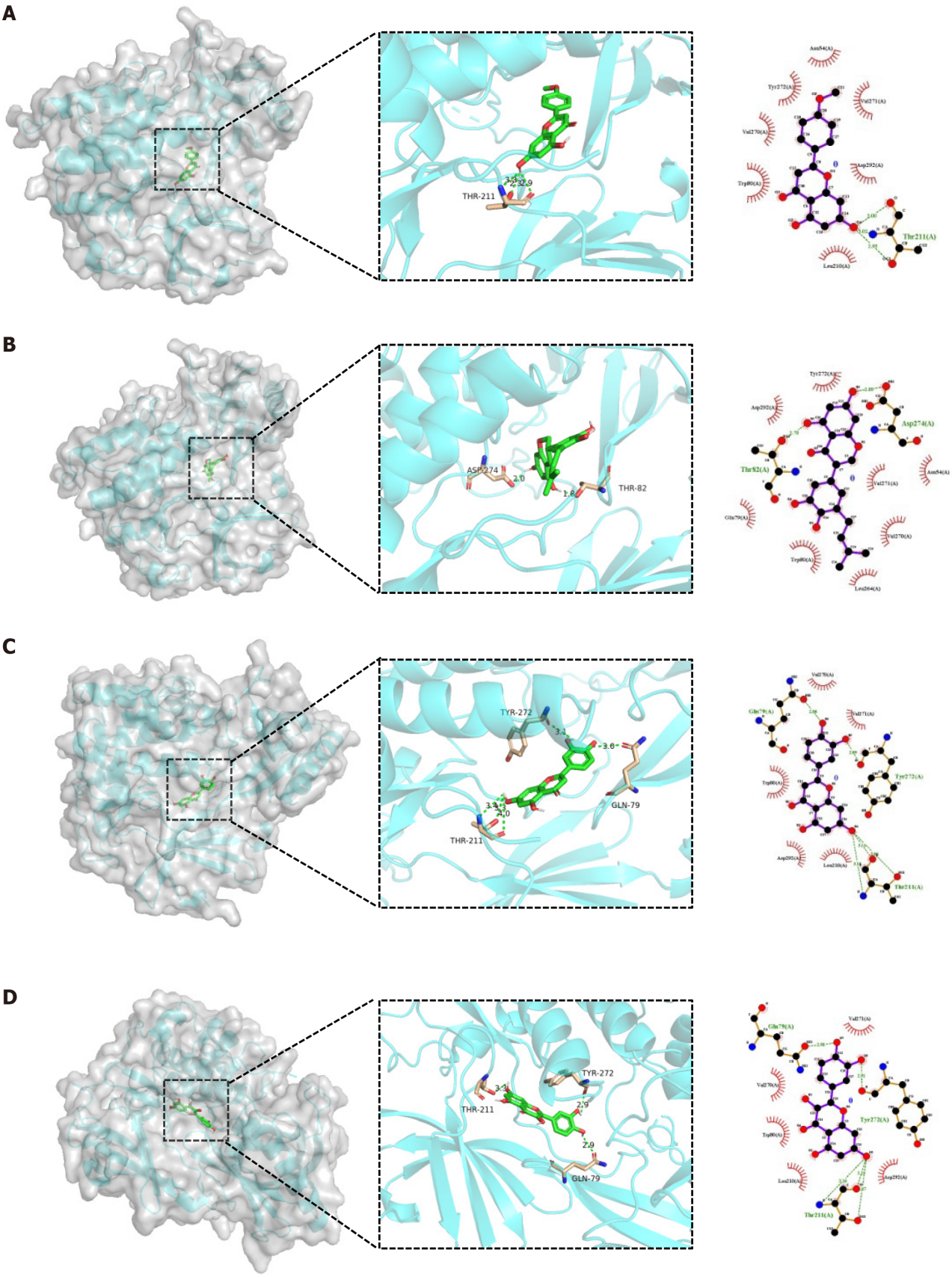

Figure 8 Molecular docking results of the core targets. A: AKT1-Acacetin; B: AKT1-Bolusanthol B; C: AKT1-Luteolin; D: AKT1-Quercetin. The amino acid residues of the human target protein, AKT1, that interacted with luteolin and quercetin were THR-211, TYR-272, and GLN-79, and those that interacted with acacetin and bolusanthol B were THR-211, ASP-274, and THR-81, where the molecules matched well with the protein cavity. In the two-dimensional diagram of molecular interactions, the green dotted lines indicate hydrogen bonding and the red dashed lines indicate hydrophobic interactions, demonstrating directionality.

Table 5 Top five core targets in the herbal-ingredient-target-pathway-disease network

Target gene	Degree	CC	BC
AR	16	0.452	0.028
CYP19A1	15	0.444	0.030
ESR2	13	0.433	0.019
PIK3R1	12	0.413	0.010
PTPN1	12	0.380	0.011

CC: Closeness centrality; BC: Betweenness centrality.

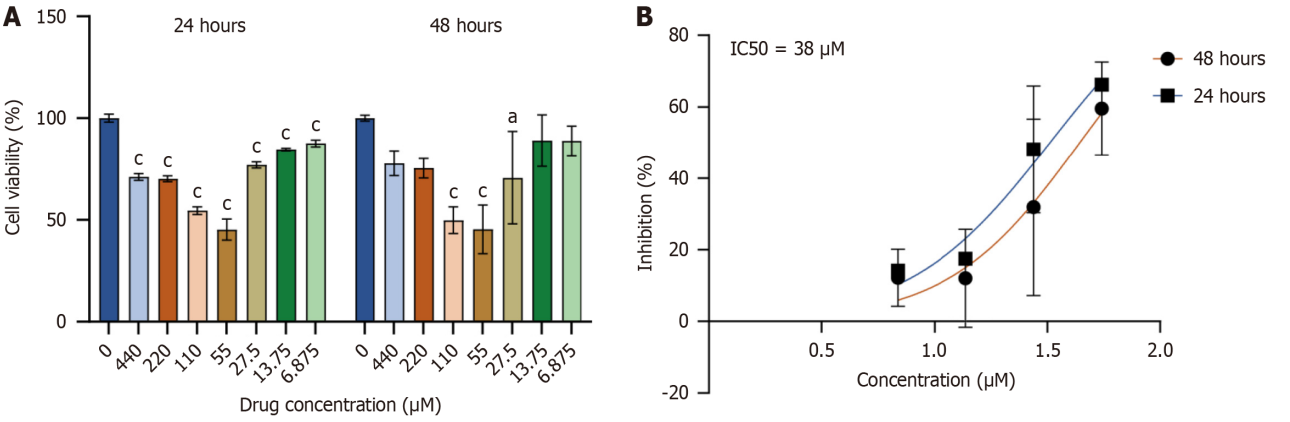

Figure 9 Acacetin markedly inhibited HT-29 cell proliferation. A: Cell viability of HT-29 cells measured with the Cell Counting Kit-8 (CCK-8) assay after treatment with different concentrations of acacetin; B: The CCK-8 assay demonstrated that the IC50 value of HT-29 cells for acacetin was 38 μM. $^{a}P < 0.05$ and $^{b}P < 0.001$ compared to control cells.

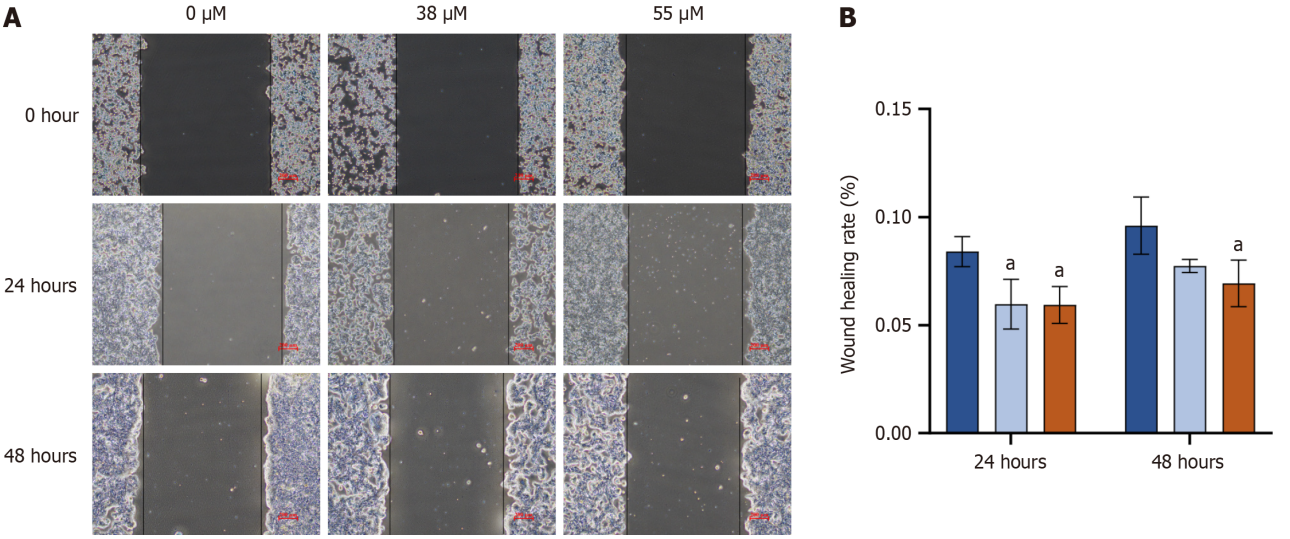

Figure 10 Acacetin suppresses HT-29 cell migration. A: HT-29 cells were treated with various doses of acacetin (0 μM, 38 μM, and 55 μM) for 24 hours and 48 hours, and their migratory behavior analyzed using wound healing assays; B: $^{a}P < 0.05$ compared to control cells.

urgent need for alternative treatments that are highly effective and have fewer adverse effects. Modern pharmacological studies and clinical trials have increasingly corroborated the clinical efficacy of Chinese herbal medicines in the prevention and treatment of CRC[32]. TCM has been shown to potentially reduce CRC risk, mitigate chemotherapy-induced adverse reactions[33], and extend the survival rate of patients with advanced CRC[34]. Exploring the molecular mechanisms involved could offer deeper insights into the role of TCM-derived molecules in CRC treatment.

Table 6 Binding energy of the molecular docking

Target	PDB ID	Ingredient	Binding energy (kcal/mol)	Hydrogen bond number	Action of amino acid residues
AKT1	6HHG	Acacetin	-9.2	1	THR-211
AKT1	6HHG	Bolusanthol B	-10.2	2	ASP-274/THR-81
AKT1	6HHG	Luteolin	-9.5	3	THR-211/TYR-272/GLN-79
AKT1	6HHG	Quercetin	-9.1	3	THR-211/TYR-272/GLN-79

Note: Binding energy function.

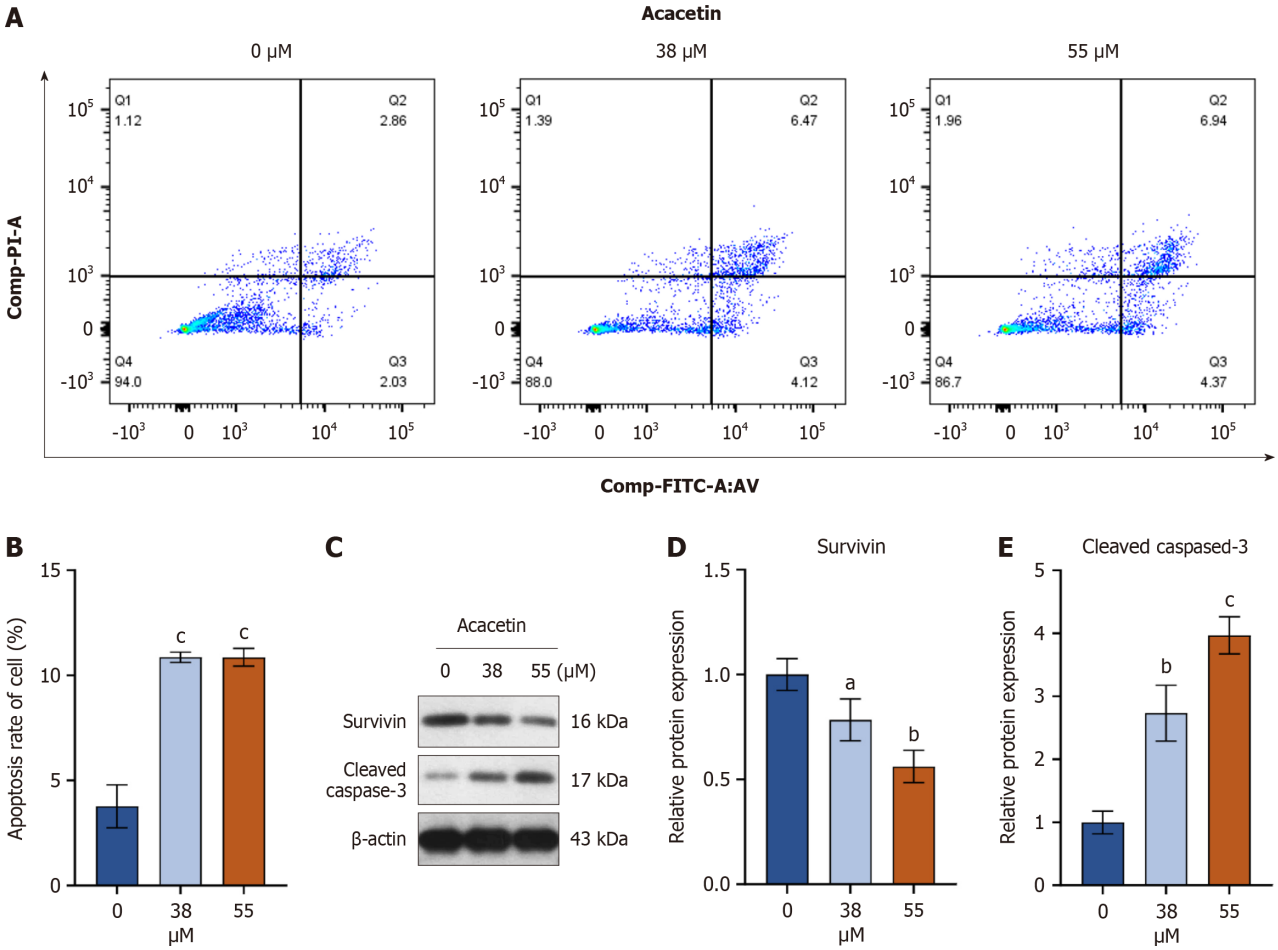

Figure 11 Acacetin promotes apoptosis in HT-29 cells. A and B: Flow cytometry using Annexin V-FITC staining detected apoptosis in HT-29 cells treated with 38 μM and 55 μM acacetin for 48 hours; C-E: Western blotting analysis evaluating apoptosis in HT-29 cells. The results showed that Acacetin (38 μM, 55 μM) could significantly increase the expression of cleaved caspase-3 protein and inhibited Survivin protein compared with the control group. The histograms indicating statistical significance levels. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

Recent studies have demonstrated the pronounced anticancer properties of *Patrinia* and Coix seed, whose extracts have been utilized in pharmacological research targeting various human cancer cell types. Specifically, the ethanol extracts of *Patrinia* yellow flowers and CSO exhibited significant anti-CRC activity in both *in vivo* and *in vitro* studies[35,36]. This study employed network pharmacology and molecular docking for the first time to identify the core components, targets, and potential mechanisms of HC therapy for the treatment of CRC. The anticancer efficacy of the core components of HC was confirmed *in vitro* using the human CRC cell line, HT-29.

The current study identified active HC components *via* the TCMSP, which were further explored using the SwissADME platform. A total of 454 drug targets were identified in the database, and a total of 2685 targets related to CRC were sourced from the GeneCards, DisGeNET, and OMTM databases. Intersecting the targets of the drug ingredients with the CRC targets yielded 171 overlapping targets. After constructing the PPI network, 30 core targets, including *EGFR*, *AKT1*, and *TNF*, among others, were identified, potentially representing the central targets of HC for CRC treatment. GO and KEGG enrichment analyses revealed the involvement of various signaling pathways, notably the PI3K/Akt signaling pathway, proteoglycans in cancer, chemical carcinogen-receptor activation, and microRNAs in

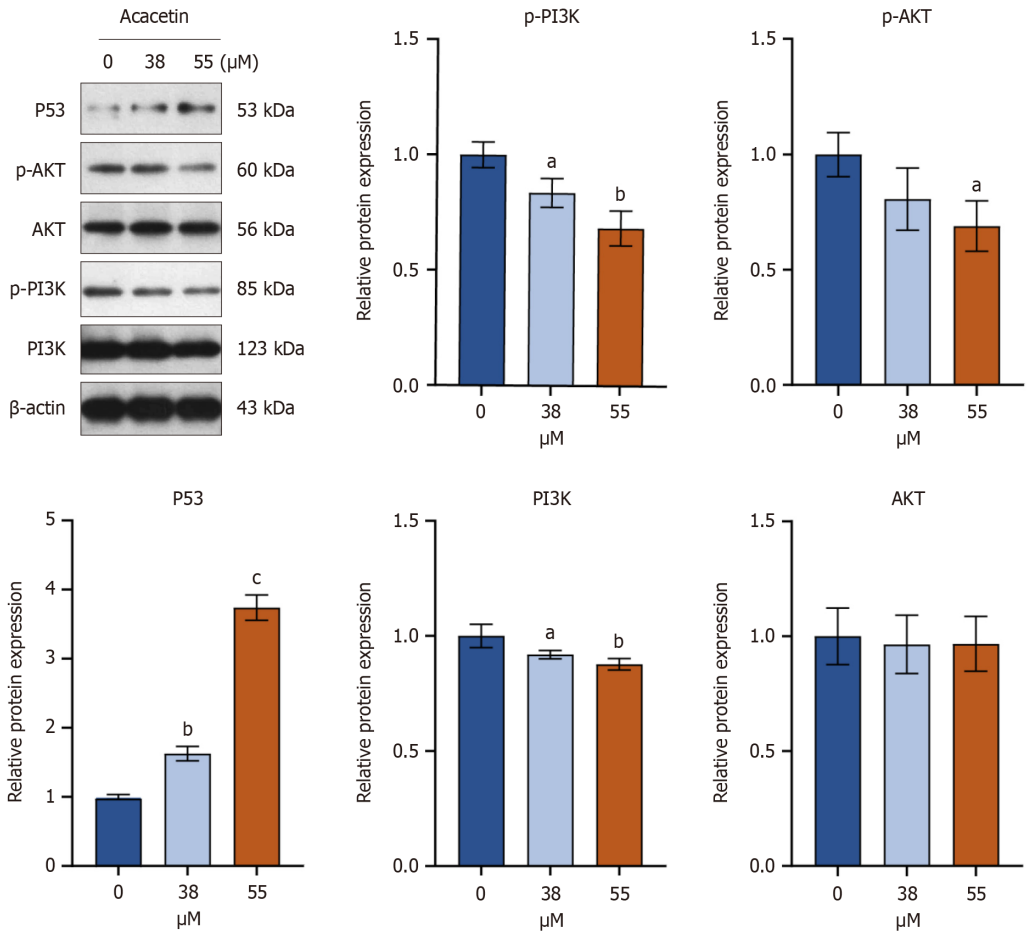

Figure 12 Acacetin inhibits HT-29 cell progression by targeting the phosphoinositide 3-kinase/Akt/p53 pathway. Western blotting analysis was conducted on HT-29 cells treated with 38 μM and 55 μM acacetin for 48 hours, with β-actin serving as the internal reference. Data quantification was performed using ImageJ software. Statistical significance was denoted as $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$. PI3K: Phosphoinositide 3-kinase.

cancer. The PI3K/Akt signaling pathway plays a pivotal role in the inhibition of CRC by HC. The PI3K/Akt pathway is implicated in the development of various cancers and influences apoptosis, autophagy, and cell survival[37]. Hyperactivity of the PI3K pathway in numerous cancers leads to reduced apoptosis and persistent proliferation[38]. Numerous oncogenes can activate *PI3K*, and its overexpression serves as a cancer biomarker[39]. Akt functions as a central kinase within the PI3K/Akt signaling pathway, enhancing the cell cycle and influencing numerous cellular functions and processes, including the p53 signaling pathway, cell proliferation, apoptosis, and the cell cycle[40]. The key anti-tumor gene, P53, which is mutated in over 50% of human cancers, loses its anti-tumor activity upon mutation, conferring carcinogenic properties to its protein[41]. Furthermore, numerous studies have indicated a strong relationship between the PI3K/Akt/p53 signaling pathway and cancer development[42], suggesting that inhibition of the PI3K signaling axis represents a promising anticancer treatment approach.

Molecular docking analysis was used to study the mechanism of action of HC on CRC. This technique effectively predicts the binding affinity between TCM components and their targets in accordance with the spatial structures of the ligands and receptors. Among the 10 targets selected for molecular docking analysis, *AKT1* was recognized as a crucial player in cell cycle regulation and PI3K/Akt signaling. Molecular docking analysis revealed that the five active ingredients in HC exhibited strong binding affinities for EGFR, AKT1, TNF, HSP90AA1, and SRC. Specifically, acacetin, bolusanthol B, luteolin, and quercetin demonstrated the most effective docking with *AKT1*. HC comprises active ingredients, such as quercetin, luteolin, and sinoacutine; however, these components are commonly found in a variety of herbs, plants, fruits, and vegetables, indicating low specificity. Utilizing Cytoscape 3.9.1, a network incorporating two herbs, 19 active ingredients, 171 intersecting targets, the top 10 pathways, and CRC diseases was constructed to illustrate the herbal-ingredient-target-pathway-disease relationship. Acacetin was identified as having the highest correlation value in this network, and the PI3K/Akt signaling pathway was identified as having the highest degree within the signaling network. Robinin, a dihydroxyl and monomethoxyflavonoid naturally found in plants, such as *Robinia*, *Damiana*, and silver birch, has shown pharmacological potential against cancer cell lines, including chemical prophylaxis and cytotoxicity[43,44]. Studies have demonstrated that acacetin exhibits anticancer properties in the colon cancer cell models, SW480 and PCT-116, by inducing mitochondrial reactive oxygen species and employing an AIF-mediated pathway[45]. Accordingly, acacetin intervention in HT-29 cells was selected to validate the predicted mechanism of action, employing various methods to corroborate the network pharmacology outcomes, such as CCK-8 for cell activity detection, Annexin V-FITC/PI labeling for apoptosis rate measurements, Transwell migration assays, and western blotting analysis. The

CCK-8 assay is frequently used to evaluate the cytotoxic potential of drugs.

This study demonstrated for the first time the cytotoxic effects of a main bioactive component of HC on HT-29 cells after 24 and 48 hours of treatment, causing inhibition of HT-29 cell proliferation. The scratch assay revealed that treatment with 38 and 55 μM acacetin significantly inhibited HT-29 cell migration. Annexin V-FITC/PI staining revealed a significant increase in the apoptosis rate of HT-29 cells after acacetin treatment. Furthermore, western blotting analysis revealed that after treatment with 55 μM acacetin, the expression level of cleaved caspase-3 significantly increased, whereas that of survivin decreased compared to those in the control group ($P < 0.05$). Caspase enzymes mediate a common pathway in apoptotic signal transduction, with numerous apoptosis-regulating pathways exerting either anti-apoptotic or pro-apoptotic effects through caspase function. Caspase-3 serves as a critical regulatory factor in tumor cell apoptosis[46]. Survivin, a tumor-specific apoptosis suppressor protein, plays a pivotal role in tumor cell differentiation, proliferation, invasion, and metastasis[47]. These findings suggested that acacetin influences HT-29 cell apoptosis by upregulating caspase-3 and downregulating survivin expression.

This study demonstrates that acacetin not only specifically binds to PI3K and Akt, but also influences their phosphorylation levels and *p53* expression. HC treatment increased *p53* expression and decreased *PI3K* and *Akt* phosphorylation in HT-29 cells. Inhibition of the PI3K/Akt signaling pathway and upregulation of p53 exhibit pro-apoptotic effects in CRC cells. Therefore, the PI3K/Akt/p53 pathway may be the principal mechanism underlying the anti-CRC effects of HC. Akt, a target activated by PI3K, serves as the primary mediator of signal transduction in the PI3K/Akt signaling pathway, and the level of Akt phosphorylation affects cell proliferation and survival[48]. The tumor suppressor gene, *P53*, which is frequently mutated in CRC, has a tumor-suppressive function that is largely attributed to the activation of target genes involved in various processes, such as cell cycle arrest, apoptosis, senescence, autophagy, ferroptosis, and cell metabolism. Hence, inhibition of p53 activity is crucial for tumorigenesis[49]. CSO, the principal component of Coix seed, has been shown to induce apoptosis in HT-29 cells by modulating the PI3K/Akt signaling pathway[50]. In summary, this study indicates that HC significantly inhibits both the growth and migration of CRC tumor cells *via* the PI3K/Akt/p53 signaling pathway.

However, this study had some limitations. Owing to the evolving nature and occasional incompleteness of database information, certain bioactive components in HC and their corresponding target proteins might not have been identified in this study. Although this preliminary study identified core targets and signaling pathways of HC for CRC treatment, the specific upstream and downstream regulatory molecules remain undefined. Further investigation into the roles of these pathways in CRC treatment is necessary. In addition, this study only conducted an *in vitro* experimental validation, and future *in vivo* animal studies are needed to further verify and support the role of acacetin in the treatment of CRC. Despite these limitations, this study provides valuable insights and evidence to advance the clinical exploration of HC for CRC treatment.

CONCLUSION

Utilizing network pharmacology, molecular docking, and *in vitro* validation, this study explored the main active ingredients of HC, as well as their mechanisms of action in treating CRC. The study findings indicate that HC could inhibit colon cancer cell proliferation, impact cell migration, and enhance apoptosis by modulating the PI3K/Akt/P53 signaling pathway. Overall, this study provides a theoretical foundation for the application of HC in the treatment of CRC.

FOOTNOTES

Author contributions: Li WD was the main contributor to study design and management; Wang CL, Yang BW, and Wang XY conducted the experiments and drafted the manuscript; Chen X, Zhai HY, and Wu Y participated in data collection and figure preparation; Cui MY, Wu JH, Meng QH, and Zhang N participated in discussions and revised the manuscript; and all authors read and approved the final manuscript.

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