

Exploration of Active Ingredients, Targets, and Potential Mechanisms of Er-Miao-San in the Treatment of Colorectal Cancer Based on Network Pharmacology and Molecular Docking Technology

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Abstract

Background: Er-Miao-San (EMS) is a classic prescription in Traditional Chinese Medicine (TCM) for the treatment of colorectal cancer (CRC) and has shown promising therapeutic effects in clinical practice. However, the specific components and molecular mechanisms of EMS remain unclear.

Purpose: The aim of this study was to analyze the effective components and molecular mechanisms of EMS in treating CRC through network pharmacology techniques and experimental validation.

Materials and Methods: The Traditional Chinese Medicine Systems Pharmacology database was used to screen the main active chemical components and targets of the EMS formula. The compound structures were verified using the PubChem database, which is an organic small-molecule bioactivity database. GeneCards and OMIM databases were utilized to predict target genes related to CRC. The Cytoscape 3.8 software was used to construct a “Drug-Active Ingredient-Target-Disease” intersection network. The STRING database was employed to analyze the core target protein–protein interaction network shared by EMS and CRC. The core targets were further subjected to Gene Ontology enrichment analysis and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis using the R language. Molecular docking between the core targets and the major active chemical components of EMS was performed using AutoDock software. The impact of the core targets on the prognosis of CRC patients was analyzed using the R language. Subsequently, we validated the potential mechanisms predicted by network pharmacology for the inhibition of CRC cell proliferation by the key proteins in the relevant pathways through CCK8 cell proliferation assays and Western blot experiments.

Results: Molecular docking results showed good docking affinity between the key active components, such as quercetin and baicalein, of EMS and the core targets. Kaplan–Meier survival analysis demonstrated a close correlation between the core targets and the survival prognosis of CRC patients. Cellular experiments showed that EMS significantly inhibited the proliferation of CRC cells and may promote apoptosis and autophagy of CRC cells by suppressing the expression of key proteins in the PI3K/AKT pathway.

Conclusion: The TCM formula EMS exerts anti-CRC effects through multiple pathways and targets, improving the prognosis and extending the survival period of CRC patients. This study provides preliminary insights into the effective components and molecular mechanisms of EMS in the treatment of CRC, which were preliminarily validated through molecular docking and experimental approaches.

Keywords

Er-Miao-San, colorectal cancer, network pharmacology, molecular docking, bioinformatics, PI3K/AKT pathway

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors in the gastrointestinal tract. According to the latest global cancer burden data released by the International Agency for Research on Cancer (IARC) of the World Health Organization in 2020, there were approximately 1.93 million new cases of CRC and 940,000 deaths globally in 2020, making it the third most common cancer and the second leading cause of cancer death, accounting for 10% and 9.4% of the total number of cancer cases and deaths, respectively. The data released by IARC show that there were approximately 550,000 new cases of CRC and nearly 300,000 deaths in China in 2020.¹ It is a major public health problem that poses a threat to human health.

The main treatments for CRC are surgery, postoperative chemotherapy, targeted therapy, and immunotherapy, but the serious side effects of drugs and drug resistance severely limit the clinical treatment of CRC.² In Asian countries, traditional Chinese medicine (TCM) has been widely accepted as an alternative therapy for malignant tumors. Clinical and pharmacological studies have shown that many natural products of Chinese herbal medicine have biological functions, such as multitarget inhibition of proliferation, promotion of cell apoptosis, inhibition of tumor invasion and metastasis, antiangiogenesis, and regulation of autophagy, reversing chemotherapy resistance *in vivo* and playing an antitumor role.^{3,4} Er-Miao-San (EMS) is a classic prescription used in TCM for the treatment of gastrointestinal tumors. It is composed of two medicines, *Atractylodes Rhizoma* and *Phellodendri chinensis* Cortex, and was first recorded in the book “Shi Yi De Xiao Fang,” which was used by ancient Chinese doctors to treat arthritis. In clinical practice, we found that EMS can effectively prolong the survival time of patients with malignant tumors and improve their quality of life. Pharmacological studies of the herbs in the EMS formula also suggest that it has pharmacological effects such as heat-clearing and detoxifying, promoting bile flow, stimulating the gastrointestinal tract, and antitumor activities.⁵

As a traditional treatment method in China, Chinese herbal medicine plays a unique role in the adjuvant treatment of CRC.⁶ However, although EMS has certain therapeutic effects in the treatment of CRC, its specific mechanism of action has not been clarified. Thanks to the rapid development of modern Chinese medicine databases and computer network topology analysis and bioinformatics, this study used network pharmacology to investigate the potential interactions between the herbs, active compounds, and potential targets in EMS, explore the binding modes and affinity between active compounds and potential targets through molecular docking technology, and to obtain a clearer understanding of the mechanism of action. We also performed experimental verification of the potential mechanism of inhibiting colon

cancer of critical proteins in relevant pathways predicted by network pharmacology to provide reference and research ideas for its clinical application and further mechanism research.

Materials and Methods

Screening of Active Ingredients and Potential Targets of EMS

The identification of effective active ingredients within EMS was conducted utilizing the Traditional Chinese Medicine Systems Pharmacology Database (TCMSP) (<https://www.tcm-sp-e.com/>).⁷ Criteria for screening included an oral bioavailability (OB) value $\geq 30\%$ and drug-like properties (DL) value ≥ 0.18 . Subsequently, the TCMSP database was employed to predict potential targets associated with the selected active ingredients. Targets were standardized and named using the UniProt database for subsequent analysis.

Acquisition of CRC Target Genes

CRC-related targets were retrieved and screened through the GeneCards (<https://genecards.weizmann.ac.il/v3/>),⁸ DisGeNET (<https://www.disgenet.org/home/>),⁹ and MalaCards (<https://www.malacards.org/>)¹⁰ databases using the keyword “Colorectal cancer.” Identified targets were then standardized and named using the UniProt database.

Construction and Analysis of the “Active Ingredient-Target-Disease”

Relationship Network Overlapping targets between active ingredients of EMS and CRC were identified using the Venny online platform (<http://jvenn.toulouse.inra.fr/app/index.html>) to eliminate duplicates. Subsequently, the “EMS active ingredient-potential target-CRC disease” relationship network was constructed using Cytoscape 3.8.0 software. Topological analysis, conducted with the Network Analyzer tool within Cytoscape, identified key active molecules and core targets.

Construction of Protein–Protein Interaction (PPI) Networks and Screening of Core-acting Proteins

To elucidate potential targets of EMS in CRC, a PPI analysis was executed using the STRING database. Cytoscape 3.8.0 software, in conjunction with the cytoHubba plugin, was utilized for network analysis based on a topology network algorithm, aiding in the identification of core functional targets.

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

The GO database (<http://www.geneontology.org>) integrates research results from the Biological Model Database Group and other biological databases, enabling analysis of cellular components (CC), molecular functions (MF), and biological processes (BP) projects on a large amount of genetic information. The KEGG database (<https://www.genome.jp/kegg/>) is a database for systematic analysis of gene functions, including the latest gene function annotations. Combining GO and KEGG enrichment analysis provides a macro perspective to obtain a large number of gene functional information and uncover drug-disease signaling pathways. The processed common targets were imported into the MetaScape database for GO and KEGG analysis, obtaining relevant data for CC, MF, BP, and KEGG pathways. R 3.5.0 software's ClusterProfiler R package was used for GO enrichment of core targets, while the ggplot2 R package was used for KEGG pathway enrichment analysis. Using a $p < .05$ standard, the top 39 GO terms and top 13 KEGG pathways were selected and imported into the Bioinformatics Mapping website (<http://www.bioinformatics.com.cn/>) to create bar charts and bubble maps.

Molecular Docking

Download the molecular structure of the drug from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). Use AutoDock4 to remove water, add hydrogen, and set the drug as the ligand. Configure the torsion tree automatically in the software. Retrieve the three-dimensional structure of the target protein from the protein database (RCSB) (<http://www.rcsb.org/>), and in AutoDock4, configure the protein as follows: remove water, replace it with hydrogen, and specify the protein as the receptor. Import the receptor and ligand PDBQT structures into AutoDock4, and define the molecular docking range. Adjust the center coordinates (center $x/y/z$) and box size (size $x/y/z$) parameters to ensure complete coverage of the protein by the docking box, with the target protein as the center of the grid.¹¹ Use AutoDock tools software to define the active pocket region of the protein receptor, and employ AutoDock Vina software to validate the binding of key components with the core targets through molecular docking.

Survival Analysis of Core Target Genes

Survival analysis of core target genes employed the survminer and survival R packages on the TCGA-COAD dataset. Colon cancer patients were stratified into high-expression and low-expression subgroups based on the optimal cutoff point. Overall survival (OS) indicators, risk ratio (HR) with 95%

confidence intervals, and log-rank p values were estimated, with statistical significance set at $p < .05$.

Experimental Validation

Cell Line

The human colon cancer cell lines HCT116 and LoVo were obtained from the American Type Culture Collection (ATCC, Manassas, USA). HCT116 and LoVo cells were grown in McCoy's 5a modified medium with 10% fetal bovine serum (FBS), which was prepared by ATCC (#30-2007). The cells were cultured at 37°C in a 5% CO₂ environment.

Preparation of EMS Extract

EMS recipe was obtained from Chinese Medicine Pharmacy of the Shanghai Traditional Chinese Medicine Hospital. EMS was composed of 15 g of *P. chinensis* Cortex Chinensis and 15 g of *Atractylodes lancea*. Place EMS (15 g of *P. chinensis* Cortex Chinensis and 15 g of *A. lancea*) in a round-bottom flask according to the proportions. Add 10 times the volume of 95% ethanol and reflux extract for 1.5 hours using an electric heating mantle. Filter out the residue, reduce the pressure, and concentrate the filtrate to dryness, weigh it, and calculate the extraction yield. Dry the residual herbal materials in a fume hood to remove the ethanol solvent. Extract the dried residues with 10 times the volume of distilled water in reflux for 2 rounds, each lasting 1.5 hours, starting the timing from the boiling point. Combine the extracts, reduce the pressure, and concentrate to a density of 1.05 g/mL (60°C). Tilt and pour out the concentrated extract, let it cool to room temperature (RT) and extract twice with an equal volume of ethyl acetate. Reduce the pressure and concentrate the ethyl acetate layer and water layer separately to dryness, then weigh and calculate the yield. Perform quality control by using high-performance liquid chromatography to determine the content of major components such as astragaloside IV in the EMS formula. Prior to the experiment, weigh an appropriate amount of the extracted powder, and dissolve it thoroughly in RPMI 1640 medium using methods such as vortex, ultrasound, and water bath. Filter the solution using a 0.22 μ m filter and prepare it to the desired concentration. Store it at 4°C for later use.

CCK-8 Assay

The HCT116 and LoVo cells in the logarithmic growth phase were seeded at a concentration of 8,000 cells per well in a 96-well plate. After the cells adhered, the serum-free culture medium was replaced, and the cells were starved overnight. Different concentrations of EMS extract, specifically 25 μ g/mL, 50 μ g/mL, 100 μ g/mL, 150 μ g/mL, 200 μ g/mL, and 250 μ g/mL were prepared to establish the control groups. After 48 hours, a 10% CCK-8 solution was added, and the cells were

cultured at 37°C for 1 hour. The absorbance of the HCT116 cells was measured at 450 nm using an enzyme-linked immunosorbent assay analyzer to calculate cell viability.

Western Blot Analysis

Western blot analysis was performed using primary antibodies from Abcam, including pan-AKT (ab8805), pan-AKT (phospho T308) (ab8933), interleukin (IL)-6 (ab233706), tumor necrosis factor- α (TNF- α) (ab183218), vascular endothelial growth factor A (VEGFA) (ab46154), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab59164). All primary antibodies were diluted 1:1,000, and the secondary antibody (ab6721, Abcam) was diluted 1:10,000. RIPA lysis buffer (Beyotime) containing 1% protein inhibitor and 1% phosphatase inhibitor (Beyotime) was used at a ratio of 100 μ L RIPA per 1×10^6 cells or 200 μ L RIPA per 20 mg tissue. The protein concentration was quantified using the Enhanced BCA Protein Assay Kit (Beyotime) according to the manufacturer's instructions. After electrophoresis separation on 10% SDS-PAGE (Solarbio), equal amounts of protein (25 μ g) were transferred onto the PVDF membrane (Millipore, MA, USA) by blotting. After blocking with 5% skim milk at RT for 1.5 hours, the membrane was incubated with primary antibody at 4°C overnight. Afterward, the membrane was washed with $1 \times$ TBST (Solarbio) three times and incubated with the secondary antibody at RT for 2 hours, followed by washing three times with $1 \times$ TBST. All proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore). In brief, the reaction mixture was added uniformly onto the membrane (protein surface), and the membrane (protein surface) was then gently put into a colorimeter. The film was scanned or photographed, and the band intensity was evaluated using Image Lab 3.0 software (Bio-Rad Laboratories).

Results

Screening of Active Ingredients in EMS and Prediction of Potential Targets

After screening and deduplication based on the OB and DL values in the TCMSP database, a total of 46 potentially active ingredients in EMS were obtained. Among them, 37 active ingredients were from *P. chinensis* Cortex and 9 active ingredients were from *Atractylodes Rhizoma* (Table 1). The compound structures of these active ingredients were verified using the PubChem database. By further screening in the TCMSP database, 455 potential target proteins were identified for the active ingredients of *P. chinensis* Cortex, and 67 potential target proteins were identified for the active ingredients of *Atractylodes Rhizoma*, resulting in a total of 522 target proteins. After deduplication, 197 potential target proteins for EMS were obtained. This indicates a high similarity in the target proteins between the two medicinal

ingredients of EMS, suggesting a synergistic effect. Subsequently, the relationships between EMS, active ingredients, and target proteins were imported into Cytoscape 3.8.0 software for data visualization. In the visualization, the active ingredients are represented by yellow nodes, the potential predicted target proteins are represented by green nodes, and the connections between active ingredients and target proteins indicate their interaction (Figure 1).

Screening for CRC Disease-related Targets and Targets Involved in the Treatment of CRC by EMS

In the quest for disease-related targets associated with CRC, a comprehensive search using the keyword “Colorectal cancer” in the GeneCards disease database yielded a substantial 10,746 CRC-related targets. Refinement with a stringent criterion of “Relevance Score > 10” resulted in the identification of 1,490 CRC disease-related targets. Simultaneously, the DisGeNET database contributed an additional 166 CRC-related targets, while the MalaCards database yielded 1,109 CRC-related targets, with 1,074 targets meeting the criteria of “Score > 25.” After the meticulous elimination of duplicates, a consolidated list of 1,748 CRC disease-related targets was achieved (Figure 2A).

After the intersection with the potential targets of the active components, 106 possible targets for CRC were obtained (Figure 2B).

Screening for Key Effective Ingredients of EMS in the Treatment of CRC

A comprehensive “Active Ingredient-Target-Disease” relationship network diagram was meticulously constructed utilizing Cytoscape 3.8.0 software. This network effectively illustrates the 106 potential target proteins associated with EMS in the treatment of CRC, along with their corresponding 22 active ingredients (Figure 3). In this graphical representation, red nodes signify active ingredients, yellow nodes denote CRC treatment target proteins, and the edges symbolize the interactions between active ingredients and CRC treatment target proteins. Employing the Network Analyzer tool within Cytoscape 3.8.0 software, topological analysis was conducted on the network. The outcome of this analysis highlighted quercetin, wogonin, beta-sitosterol, rutaecarpine, and palmatine as key pharmacological molecules, potentially holding central regulatory roles in the treatment of CRC with EMS (Table 1).

PPI Network Construction and Core Protein Screening

We submitted the 106 potential CRC treatment targets to STRING, a PPI database, for protein interaction analysis. Utilizing the cytoHubba plugin with the MCC algorithm in

Table 1. The Main Effective Components and Relevant Parameters of Er-Miao-San (EMS) in Treating Colorectal Cancer (CRC).

Number	Traditional Chinese Medicine Systems Pharmacology (TCMSP) ID	Main Active Ingredients	Name	Source	Degree Value	Oral Bioavailability (OB)/%	Drug-like Properties (DL)
1	MOL000098	Quercetin	Meletin	<i>P. chinensis</i> Cortex	75	46.43	0.28
2	MOL000173	Wogonin	Wogonin	<i>Atractylodes Rhizoma</i>	33	30.68	0.23
3	MOL000358	Beta-sitosterol	Cupreol	<i>P. chinensis</i> Cortex	14	36.91	0.75
4	MOL002662	Rutaecarpine	Rutacarpine	<i>P. chinensis</i> Cortex	13	40.3	0.6
5	MOL000785	Palmatine	Fibrauretin	<i>P. chinensis</i> Cortex	11	64.6	0.65
6	MOL001454	Berberine	Berberine	<i>P. chinensis</i> Cortex	9	36.86	0.78
7	MOL002894	Berberrubine	Berberubine	<i>P. chinensis</i> Cortex	9	35.74	0.73
8	MOL006422	Thalifendine	Fen's pine pine	<i>P. chinensis</i> Cortex	9	44.41	0.73
9	MOL001458	Coptisine	Coptisine	<i>P. chinensis</i> Cortex	7	30.67	0.86
10	MOL000449	Stigmasterol	Stigmasterol	<i>P. chinensis</i> Cortex	7	43.83	0.76
11	MOL002668	Worenine	Worenine	<i>P. chinensis</i> Cortex	6	45.83	0.87
12	MOL002666	Chelerythrine	Chelerythrine	<i>P. chinensis</i> Cortex	4	34.18	0.78
13	MOL002651	Dehydrotanshinone II A	1,2-Didehydrotanshinone IIA	<i>P. chinensis</i> Cortex	4	43.76	0.4
14	MOL000787	Fumarine	Fumarine	<i>P. chinensis</i> Cortex	4	59.26	0.83
15	MOL001131	Phellamurin_qt	Dihydroxanthine	<i>P. chinensis</i> Cortex	4	56.6	0.39
16	MOL000188	3 β -Acetoxyatractylone	Acetyl one	<i>Atractylodes Rhizoma</i>	4	40.57	0.22
17	MOL000790	Isocorypalmine	Isocorypalmine	<i>P. chinensis</i> Cortex	3	35.77	0.59
18	MOL001455	(S)-Canadine	Tetrahydroberberineper	<i>P. chinensis</i> Cortex	2	53.83	0.77
19	MOL002670	Cavidine	Kavidin	<i>P. chinensis</i> Cortex	2	35.64	0.81
20	MOL002644	Phellopterin	Phellopterin	<i>P. chinensis</i> Cortex	2	40.19	0.28
21	MOL001771	Poriferast-5-en-3 β -ol	Porous steroid-5-en-3 β -ol	<i>P. chinensis</i> Cortex	2	36.91	0.75
22	MOL002663	Skimmianin	Skimmianine	<i>P. chinensis</i> Cortex	2	40.14	0.2



significance of these proteins in the regulatory network. Through the MCC algorithm, several protein nodes, including VEGFA, AKT1, TNF, IL6, and PTGS2, exhibited higher parameters, suggesting their potential core role in the treatment of CRC with EMS (Figure 4B).

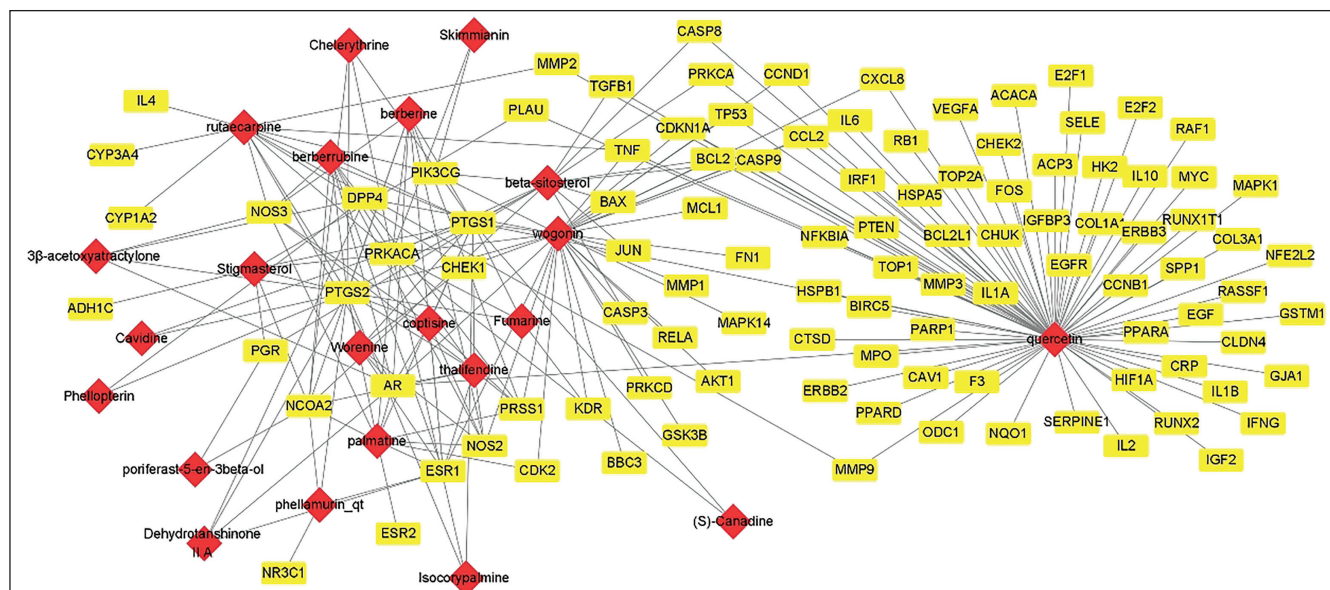


Figure 3. Network Diagram of the Relationship between Active Components, Therapeutic Targets, and Disease.

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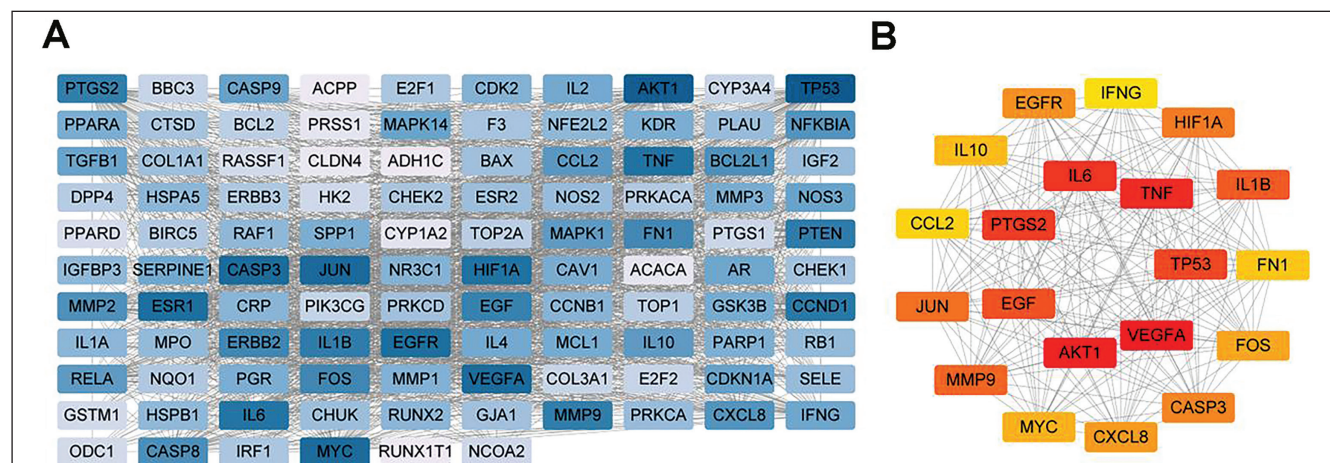


Figure 4. (A) Protein-Protein Interactions (PPIs) of Er-Miao-San (EMS's) Anti-colorectal Cancer (CRC) Targets Using PPI Network Analysis. (B) Top 20 Core Targets Identified by Topological Network Analysis.

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Biological Function Enrichment Analysis

To delve deeper into the biological functions of EMS in treating CRC, we conducted a comprehensive biological function enrichment analysis of the 106 target proteins. Employing the ClusterProfiler and ggplot2 packages in R 3.5.0 software, we set a filtering criterion of $p < 0.05$ to analyze and visualize potential biological functions (Figure 5). The GO enrichment analysis, focusing on BP, illuminated that EMS may exert its anti-CRC effects through various BP. These include oxidative stress, lipopolysaccharide response, bacterial response, apoptosis, steroid hormone response, and epithelial cell proliferation. In the CC analysis, target proteins implicated in the anti-CRC effects of EMS

were predominantly enriched in cellular membrane components (membrane rafts, membrane microdomain, membrane region), transcription factor complexes, RNA polymerase II transcription factor complexes, luminal structures, mitochondrial outer membranes, and nuclear chromatin. The MF analysis suggested that EMS is also involved in molecular processes such as ubiquitin-protein ligase activity, DNA-binding transcription activator, RNA polymerase II, serine/threonine kinase activity, cytokine (ligand/receptor) activity, and peptidase activity. Furthermore, KEGG pathway enrichment analysis unveiled associations between EMS therapeutic effects and several signaling pathways, including cancer, immune system, endocrine metabolism, and apoptosis. Notable pathways encompassed

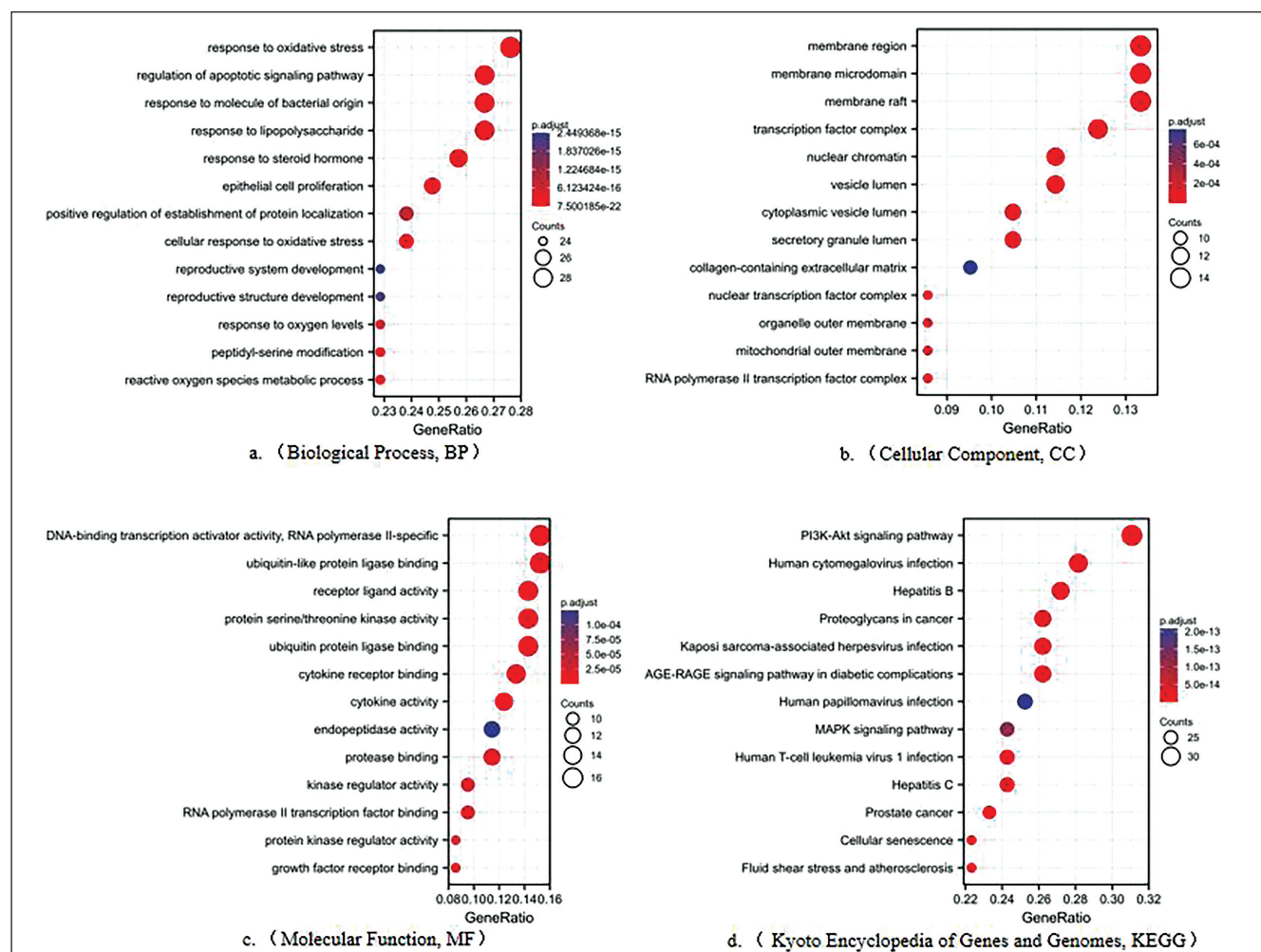


Figure 5. Biological Function Enrichment Analysis of Er-Miao-San (EMS) Target Proteins in Anti-colorectal Cancer (CRC).

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the PI3K-AKT signaling pathway, protein-polysaccharide in cancer, mitogen-activated protein kinase (MAPK) signaling pathway, cell senescence, IL17 signaling pathway, endocrine resistance, and apoptosis pathways.

Docking Analysis of Key Components in EMS and Core Target Proteins

To establish the connection between the key components of EMS and the core targets, we obtained the protein crystal structures corresponding to the targets from the PDB database. Additionally, we consulted relevant literature to understand the protein active sites for VEGFA,¹² AKT1,¹³ TNF,¹⁴ and IL6.¹⁵ Based on this information, we determined the protein pockets (Table 2). We conducted computer-simulated molecular docking verification between the key components, quercetin, and wogonin of two Chinese medicines, *P. chinensis* Cortex and *Atractylodes Rhizoma*, and the top four core targets VEGFA, AKT1, TNF, and IL6 in topological network analysis.¹² The results of the docking simulations

demonstrated that both quercetin and wogonin exhibited commendable binding abilities with VEGFA, AKT1, TNF, and IL6 (Table 3 and Figure 6). Affinity values are indicative of the binding performance between the ligand and receptor proteins. A lower affinity value suggests a more favorable interaction between the molecule and protein. An affinity value less than 0 indicates spontaneous binding of the ligand molecule to the receptor protein, while a value smaller than -5.0 kJ mol^{-1} signifies excellent binding between the molecule and the protein.

In Figure 6, the blue part represents the drug molecule, the yellow part represents the target protein structure, the left figure represents the overall binding pattern between the two, and the right represents the enlarged picture of molecule and protein binding.

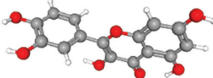
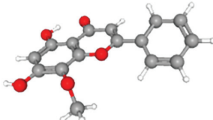
Survival Analysis of Core Target Genes

To further analyze the association of EMS anti-CRC core targets with CRC disease prognosis. Utilizing R 3.5.0

Table 2. Target Gene Protein Pocket Coordinates and Grid Box Sizes.

Target Gene	PDB ID	Protein Pocket Coordinates	Grid Box Size
VEGFA	1BJI	$x = 31.702, y = -26.149, z = -15.341$	$x = 22.0, y = 28.0, z = 28.0$
AKT1	6HHH	$x = 20.528, y = 11.028, z = 28.722$	$x = 28.0, y = 34.0, z = 32.0$
TNF	5M2I	$x = 11.976, y = -25.572, z = -104.917$	$x = 24.0, y = 22.0, z = 16.0$
IL6	4NI7	$x = -2.969, y = 3.932, z = 11.499$	$x = 24.0, y = 18.0, z = 26.0$

Table 3. Molecular Docking Results of Key Components and Core Target Proteins of Er-Miao-San (EMS).

Compound	3D Structure	Target	PDB Protein Identifier	Binding Energy/kJ mol ⁻¹
Quercetin		VEGFA	1BJI	-5.3
		AKT1	6HHH	-6.5
		TNF	5M2I	-5.7
		IL6	4NI7	-5.7
Wogonin		VEGFA	1BJI	-5.0
		AKT1	6HHH	-8.8
		TNF	5M2I	-5.7
		IL6	4NI7	-5.9

software along with the survminer and survival R packages, we examined the molecular expression patterns in CRC clinical patients sourced from the TCGA-COAD dataset. Employing the optimal cutoff point, CRC patients were stratified into high and low expression subgroups for the core target genes, with a significance threshold of $p < 0.05$. Subsequently, the survival prognostic significance of these core target genes in CRC was analyzed based on the OS indicator. The results unveiled that among the top eight core target genes VEGFA, TNF, PTGS2, TP53, EGF, and other targets exhibited noteworthy prognostic significance in CRC (Figure 7), indicating that EMS may exert a therapeutic role in prolonging the survival of CRC patients by targeting relevant targets.

Validation of the Colorectal Cancer Effect of EMS *in vitro*

To further investigate the inhibitory effect of EMS on CRC cell proliferation, we performed *in vitro* experiments. HCT116 and LoVo cells were treated with increasing concentrations of EMS for 24 hours, and cell viability was measured. As shown in Figure 8, EMS significantly inhibited the growth of HCT116 (Figure 8A) and LoVo cells (Figure 8B), with IC₅₀ values of $122.47 \pm 3.432 \mu\text{g/mL}$ and $98.432 \pm 5.891 \mu\text{g/mL}$, respectively, indicating a good inhibitory effect of EMS on the proliferation of CRC cells.

The network pharmacology and molecular docking results suggested that the PI3K/AKT signaling pathway might play an important regulatory role in the process of EMS inhibiting CRC cell proliferation, we further conducted immunoblot

analysis. The results showed that after EMS intervention, the phosphorylation levels of the key molecules of the PI3K/AKT signaling pathway were significantly inhibited in HCT116 and LoVo cells, along with downregulation of core molecules such as VEGFA, TNF, and IL6 (Figure 8C-F). These findings indicate that EMS promotes apoptosis and autophagy levels in HCT116 and LoVo cells through the PI3K/AKT signaling pathway.

Discussion

CRC is one of the malignant tumors that seriously damage human life. In recent decades, its incidence and mortality have been on the rise. TCM, as a unique treatment method in China, has shown great advantages in prolonging the survival time and improving the quality of life of patients with CRC. Modern pharmacological studies have found that two Chinese herbal medicines, *P. chinensis* Cortex and *Atractylodes Rhizoma*, in EMS have pharmacological effects such as clearing heat and detoxification, promoting bile flow, stimulating the gastrointestinal tract, and antitumor, but their specific mechanisms of action are still unclear.

This study conducted computational analysis through pharmacology, disease databases based on network pharmacology, bioinformatics, and topology algorithms to discover that 22 key pharmacological molecules in EMS may exert anti-CRC therapeutic effects through 106 targets. The BP involved include oxidative stress, cell apoptosis, cell proliferation, and tumor metabolism, and the treatment effects are exerted through pathways such as regulating the PI3K-AKT signaling pathway, the cancer protein-polysaccharide

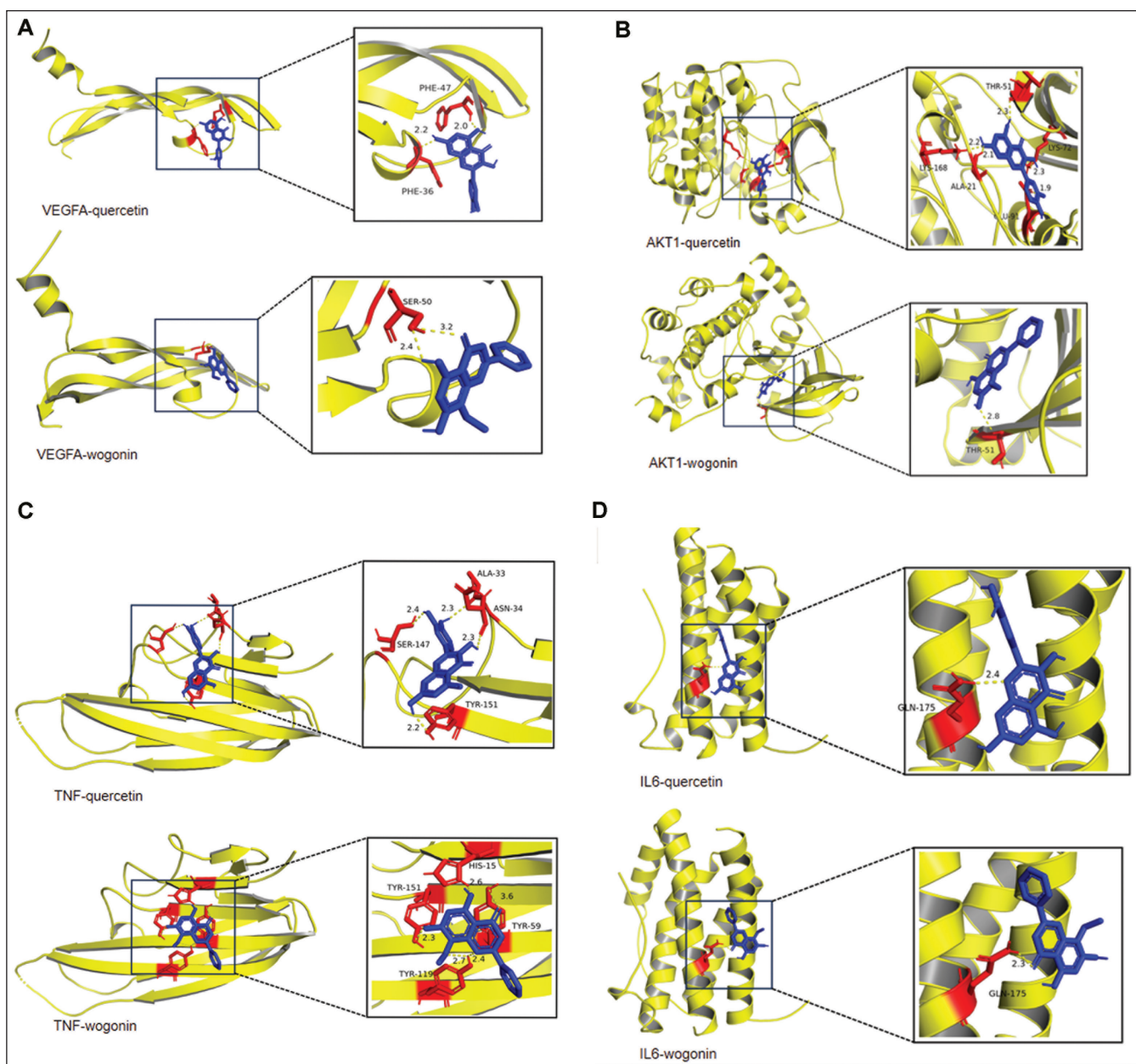


Figure 6. Combination of the Main Compounds and Protein Targets (A–D).

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signaling pathway, and the MAPK signaling pathway. Among the 22 key pharmacological molecules, quercetin, wogonin, beta-sitosterol, rutaecarpine, and palmatine may be the key pharmacological molecules in the treatment of CRC with EMS. Specifically, quercetin has been shown to have multiple anti-tumor effects, including inhibiting tumor cell proliferation, reversing tumor cell multidrug resistance, and enhancing antitumor effects when combined with other agents.¹⁶ Wogonin is a natural flavonoid compound that can promote tumor cell apoptosis, block the cell cycle of tumor cells, inhibit tumor angiogenesis, increase the infiltration of immune cells, and enhance immune function.^{17,18}

Beta-sitosterol is a steroid-like plant oil with various pharmacological activities. It can inhibit tumor occurrence and development, induce tumor cell differentiation, stimulate tumor cell apoptosis, play an antioxidant role, and inhibit tumor cell proliferation by activating the sphingomyelin cycle.¹⁹ Rutaecarpine is a naturally occurring indole alkaloid that can inhibit the proliferation of a variety of tumor cells, including cervical cancer, colon cancer, lung cancer, melanoma, T lymphocytic leukemia, prostate cancer, and breast cancer.²⁰ Palmatine is a bioactive alkaloid that can enhance immune function by regulating cytokines and immune cells.²¹

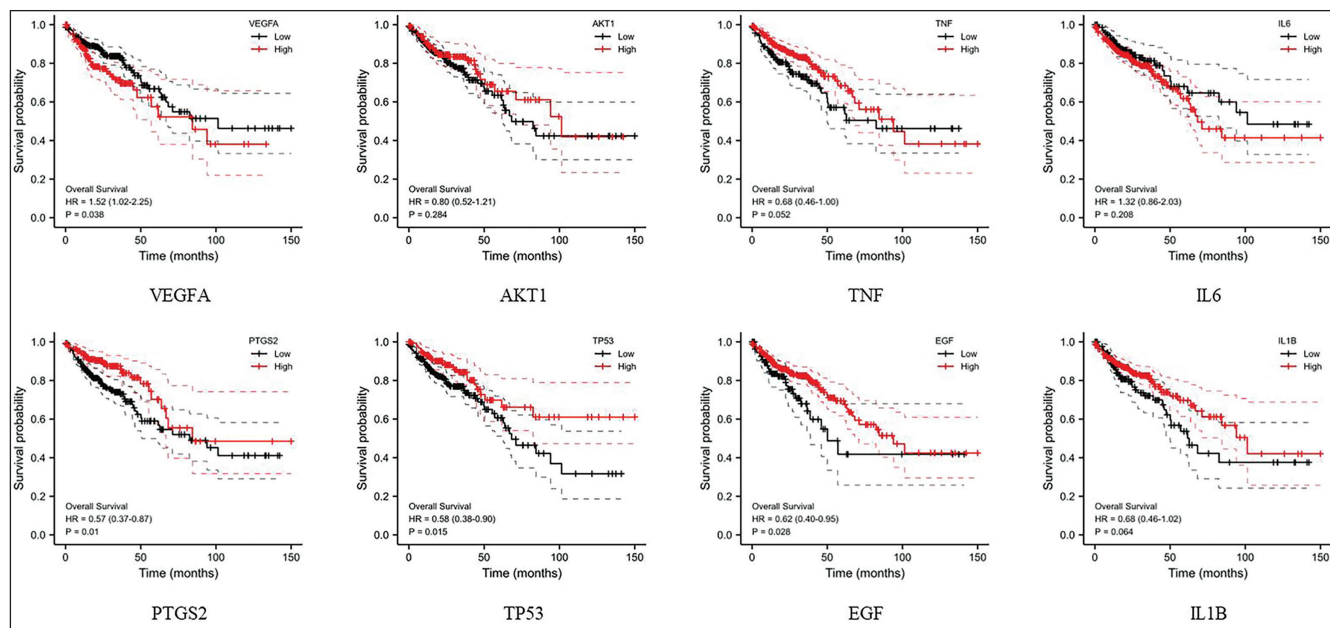


Figure 7. Survival Analysis of the Core Target of Er-Miao-San (EMS) in Resistance to Colorectal Cancer (CRC).

Note: For color usage in this figure, please refer to the web version of the article.

Based on our predictions of active ingredient targets and “active ingredient-potential target-disease” relationship network construction as well as PPI analysis, VEGFA, AKT1, TNF, IL6, PTGS2, TP53, EGF, IL1B, and other core targets may play critical regulatory roles in the anti-CRC effects of EMS. Tumor proliferation and metastasis rely on the blood vessels generated to provide nutrients. Therefore, blocking tumor angiogenesis is currently a hot topic in tumor therapy. VEGFA, as a vascular endothelial growth factor, increases tumor vascular permeability, promotes the proliferation of endothelial cells, and induces tumor angiogenesis when highly expressed.²² Clinical studies have confirmed that anti-VEGFA drugs have good therapeutic effects in CRC treatment. EGF, as a growth factor, can promote the growth and proliferation of epithelial cells and is an important messenger for signal communication between tumor cells and matrix cells. TNF, IL6, and IL1B, as inflammatory cytokines, play different roles in inflammation, cell differentiation, lipid metabolism, and cell apoptosis. PTGS2 has been found to be highly expressed in many benign cancer precursors and malignant tumors can promote tumor angiogenesis, enhance tumor cell invasion and metastasis, and inhibit cell apoptosis.^{23,24} AKT1 is a key molecule in the PI3K-PTEN-AKT-mTOR signaling pathway and plays an important regulatory role in tumor cell growth, apoptosis, invasion, and metabolism.²⁵ TP53 is a tumor suppressor gene. Mutations of TP53 are widely present in many types of cancer cells. When this gene is mutated, p53 becomes inactive, leading to the formation of tumors.²⁶

Through GO function enrichment and KEGG pathway enrichment analysis, we found that EMS may exert various biological functions through signaling pathways such as PI3K-Akt signaling pathway, cancer protein-polysaccharide signaling pathway, MAPK signaling pathway, cell senescence pathway, IL17 signaling pathway, endocrine resistance pathway, and apoptosis pathway in cancer treatment, immune regulation, endocrine metabolism, tumor cell apoptosis, etc. Combined with our molecular docking verification, the key active ingredients in EMS, quercetin, and wogonin, have good binding ability against the core targets screened from the PPI network. Moreover, most of the targets in the anti-CRC core targets of EMS are also meaningful in CRC prognosis, indicating that EMS may exert a therapeutic effect on CRC patients’ survival by targeting relevant targets. Especially, the *in vitro* experimental results show that EMS can significantly inhibit the phosphorylation of key molecules in the PI3K/AKT signaling pathway and downregulate core molecules such as VEGFA, TNF, and IL6.

In summary, based on our network pharmacology and molecular docking studies, the key bioactive ingredients of EMS, quercetin, wogonin, etc., through signaling pathways such as PI3K-Akt, cancer protein-polysaccharide, and MAPK, regulate core targets such as VEGFA, AKT1, TNF, and IL6, to exert therapeutic effects for cancer treatment, immune regulation, endocrine metabolism, and tumor cell apoptosis. This provides research direction and reference for future clarification of the specific effective ingredients and mechanism of action of EMS in the treatment of CRC.

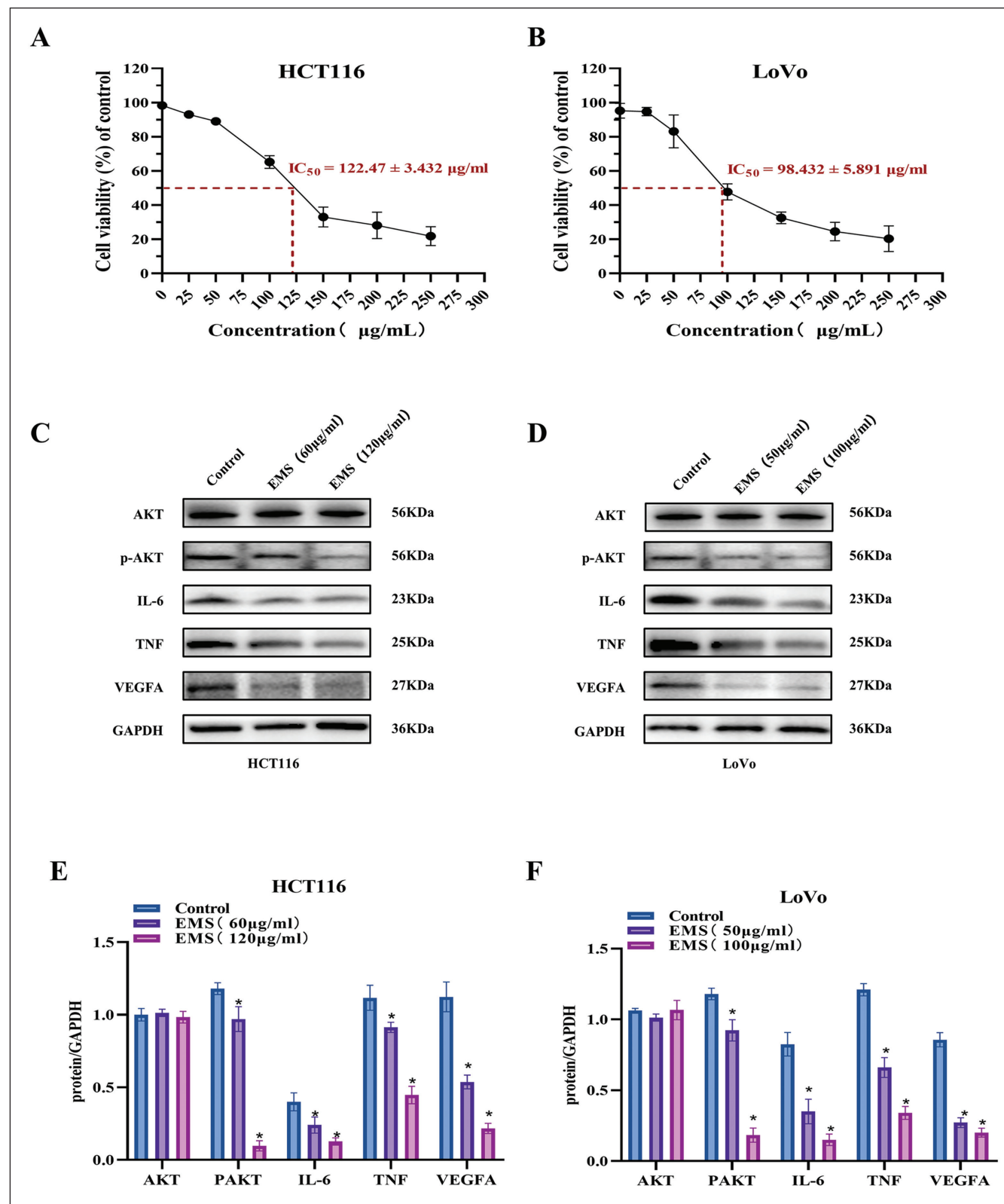


Figure 8. Anti-proliferative Effect of Er-Miao-San (EMS) and Immunoblot Analysis of Four Core Molecules after Treatment with EMS for 24 hours in HCT116 (A, C, and E) and LoVo (B, D, and F).

Note: For color usage in this figure, please refer to the web version of the article.

Conclusion

EMS, a classical Chinese medicine formula, emerges as a promising therapeutic approach in the fight against CRC. Its effectiveness is evidenced by its ability to influence diverse pathways and targets, ultimately enhancing the prognosis, and extending the survival of individuals grappling with CRC. This study provides bioinformatic insights that could pave the way for a deeper understanding of the mechanisms of action of the EMS.

This comprehensive analysis not only affirms EMS efficacy but also lays the groundwork for further exploration into the intricate workings of this classical Chinese medicine. By elucidating the complex interplay between EMS and the biological pathways associated with CRC, this study not only validates its therapeutic potential but also offers a roadmap for future research endeavors.

Abbreviations

CRC: Colorectal cancer; **EMS:** Er-Miao-San; **TCM:** Traditional Chinese Medicine; **IARC:** International Agency for Research on Cancer; **TCMSP:** Traditional Chinese Medicine Systems Pharmacology; **PPI:** Protein–protein interaction; **GO:** Gene Ontology; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **OB:** Oral bioavailability; **DL:** Drug-like properties.

Authors' contributions

Jianlin Ren and Jing Lin conceived and designed the study. Chenyue Yuan, Yong Zhao, and Yuting Yan performed the data analysis. Lin Zhou and Jinyan Chu wrote the paper. All authors have read and approved the final manuscript.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval and Informed Consent

Not applicable.

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