

Network Pharmacology Prediction and Experimental Validation of Apocynin Against Colon Adenocarcinoma: Identification of CCND1 and CXCL8 as Key Targets

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Abstract

Background

Colon adenocarcinoma (COAD) remains a leading cause of cancer-related mortality, necessitating novel therapeutic strategies. Apocynin, a natural NADPH oxidase inhibitor, exhibits anti-tumor potential, yet its precise mechanisms against COAD remain poorly characterized.

Methods

An integrative approach combining, bioinformatics, molecular docking, and in vitro experimental validation was employed. Apocynin targets and COAD differentially expressed genes (DEGs) were retrieved from GeneCards and TCGA databases, respectively. Protein-protein interaction (PPI) networks, functional enrichment, molecular docking, and survival analyses were performed. Phenotypic effects—including apoptosis, cell cycle progression, invasion, and mitotic activity—were evaluated in colon cancer cells treated with Apocynin (APO), hyperthermia (HT), or their combination (HT + APO).

Results

58 intersecting targets were identified between Apocynin and COAD DEGs, from which seven hub genes (ALB, CCND1, CXCL12, CXCL8, SERPINE1, SPP1, TIMP1) were extracted. KEGG enrichment predominantly highlighted the PI3K/Akt signaling pathway. Molecular docking confirmed robust binding affinities, with CCND1 exhibiting the strongest interaction (-6.8 kcal/mol). Survival analysis identified CXCL8 as a significant adverse prognostic biomarker ($P = 0.00048$). In vitro, Apocynin induced apoptosis, triggered G0/G1 cell cycle arrest, suppressed invasion, and reduced the mitotic index. Furthermore, the HT + APO combination demonstrated pronounced synergistic anti-tumor effects across all phenotypic assays.

Conclusions

Apocynin exerts its anti-COAD effects primarily through the regulation of CCND1 and CXCL8. The synergistic efficacy of Apocynin combined with hyperthermia provides a compelling rationale for its clinical development as a combinatorial therapeutic strategy for colon adenocarcinoma.

1. Introduction

Colon adenocarcinoma (COAD), the predominant histological subtype of colorectal cancer, accounts for approximately 1.9 million new cases and 935,000 deaths annually worldwide, representing a substantial global health burden^{1,2}. Despite advances in surgical resection, cytotoxic chemotherapy, and molecularly targeted agents, the five-year survival rate for patients with metastatic disease remains below 15%,

primarily attributable to acquired drug resistance and systemic toxicity^{3,4}. These clinical limitations underscore the urgent need for novel therapeutic agents with improved efficacy profiles and reduced adverse effects.

Natural products have historically served as a cornerstone for anticancer drug discovery, offering structurally diverse compounds with multi-target pharmacological activities⁵. Apocynin (4-hydroxy-3-methoxyacetophenone), a methoxy-substituted catechol originally isolated from *Apocynum cannabinum*, is a well-characterized inhibitor of NADPH oxidase (NOX)⁶. While traditionally investigated for its anti-inflammatory and antioxidant properties in cardiovascular and neurodegenerative diseases, emerging evidence indicates that Apocynin possesses significant anti-tumor activity across multiple malignancies. Mechanistic studies have demonstrated that Apocynin suppresses cancer cell proliferation, migration, and invasion through modulation of reactive oxygen species (ROS) levels, inhibition of NF- κ B signaling, and induction of cell cycle arrest in breast cancer, lung cancer, and glioma models^{7,8}. However, the comprehensive molecular mechanisms underlying its anti-COAD effects remain poorly characterized, and systematic investigations integrating computational prediction with experimental validation are currently lacking. Network pharmacology has emerged as a transformative paradigm for elucidating the polypharmacological mechanisms of natural compounds^{9,10}. By constructing and analyzing drug-target-disease interaction networks, this systems biology approach enables the identification of critical molecular hubs and signaling pathways, thereby providing a mechanistic framework for understanding multi-target drug actions. Given that natural products typically exert therapeutic effects through simultaneous modulation of multiple targets rather than single receptor interactions, network pharmacology is particularly well-suited for mechanistic dissection of compounds such as Apocynin.

In the present study, we employed an integrated strategy combining molecular docking, and in vitro experimental validation to systematically investigate the anti-COAD mechanisms of Apocynin. We identified overlapping targets between Apocynin and COAD, constructed protein-protein interaction networks to extract hub genes, performed functional enrichment and survival analyses, and validated drug-target interactions through molecular docking. Furthermore, we conducted flow cytometry, Transwell invasion assays, and immunofluorescence staining to experimentally confirm the predicted anti-tumor effects of Apocynin and its combination with hyperthermia in colon cancer cells. Our findings provide mechanistic insights into the anti-COAD activity of Apocynin and establish a rationale for its potential clinical application, either as monotherapy or in combination with thermal ablative strategies.

2. Materials and Methods

2.1 Data Sources and Target Gene Collection

Transcriptomic data for colon adenocarcinoma (COAD) were sourced from The Cancer Genome Atlas (TCGA) database (<https://portal.gdc.cancer.gov/>)¹¹. RNA-seq transcriptomic data (HTSeq-Counts) of

COAD, including tumor tissue samples and paired normal tissue samples, were downloaded from this database for subsequent differential expression analysis.

2.2 Screening of Differentially Expressed Genes

Differential expression analysis was performed on the TCGA-COAD dataset using the DESeq2 package (version 1.40.0) in R software (version 4.3.0), with thresholds of $|\log^2\text{FoldChange}| > 1.5$ and Benjamini-Hochberg adjusted P-value < 0.05 to identify differentially expressed genes (DEGs)¹². The intersection of the 303 Apocynin target genes and the TCGA-COAD DEGs was determined using the VennDiagram package in R¹³. A Venn diagram was plotted to visualize the overlapping genes, which were defined as the potential core target genes of Apocynin against colon cancer.

2.3 Construction of PPI Network and Hub Gene Identification

The overlapping genes were imported into the STRING database (<https://string-db.org/>) to construct a protein-protein interaction (PPI) network, with the organism set to Homo sapiens and the minimum required interaction score set to 0.400 (high confidence)¹⁴. The PPI network was visualized and analyzed using Cytoscape software (version 3.9.1). The CytoHubba plugin, was used to identify the top-ranked hub genes within the PPI network¹⁵. The core subnetwork was extracted for further analysis.

2.4 GO and KEGG Enrichment Analysis

Gene Ontology (GO) enrichment analysis, including biological process (BP), cellular component (CC), and molecular function (MF) categories, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed on the overlapping genes using the clusterProfiler package (version 4.8.0) in R. The Benjamini-Hochberg method was applied for multiple testing correction, with statistical significance defined as adjusted P-value < 0.05 . The top 10 terms in each GO category and the top 20 KEGG pathways were visualized using bar plots and bubble plots, respectively.

2.5 Molecular Docking

The three-dimensional structure of Apocynin was retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) in SDF format and converted to PDB format using OpenBabel software¹⁶. The crystal structures of the hub gene-encoded proteins were downloaded from the Protein Data Bank (PDB) database (<https://www.rcsb.org/>)¹⁷. Protein structures were preprocessed by removing water molecules and heteroatoms, adding hydrogen atoms, and calculating charges using AutoDockTools (version 1.5.7)¹⁸. Molecular docking was performed using AutoDock Vina (version 1.2.3), with the search space (grid box) centered on the binding pocket coordinates (x, y, z dimensions of $60 \times 60 \times 60$ Å, grid spacing 0.375 Å) defined to encompass the active site of each protein. For proteins without known ligand-bound structures, the grid box was centered on the geometric centroid of the predicted active site using CASTp server. The binding affinity (kcal/mol) was recorded, and the optimal

docking pose was selected based on the lowest binding energy. The results were visualized using PyMOL and LigPlot+ software¹⁹.

2.6 Survival Analysis

Survival analysis of the hub genes was performed using the survival and survminer packages in R. Patients with COAD from the TCGA dataset were stratified into high-expression and low-expression groups based on the median expression level of each hub gene. Kaplan-Meier overall survival curves were generated, and the log-rank test was applied to assess the statistical significance of survival differences between the two groups. A P-value < 0.05 was considered statistically significant.

2.7 Cell Culture and Treatment

Human colon cancer cell lines HCT116 and SW480 (obtained from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Cells were randomly assigned to four experimental groups: (1) Control group (untreated cells), (2) HT group (cells treated with hyperthermia), (3) APO group (cells treated with Apocynin), and (4) HT + APO group (cells treated with the combination of hyperthermia and Apocynin). Apocynin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO to prepare a 100 mM stock solution and stored at -20°C. For experiments, cells were treated with Apocynin at 600 µM, with the final DMSO concentration not exceeding 0.1% (v/v). A vehicle control group containing 0.1% DMSO without Apocynin was included in all experiments to exclude solvent effects. Hyperthermia treatment was administered by incubating cells in a water-jacketed CO₂ incubator at 43.0 ± 0.2°C for 1 hour per session, repeated once daily for three consecutive days. The control group was maintained at 37°C throughout the experiment. All cell lines were authenticated by short tandem repeat (STR) profiling within the last 12 months and tested negative for mycoplasma contamination.

2.8 Flow Cytometry for Apoptosis Analysis

Cell apoptosis was assessed using the Annexin V-FITC/PI double staining kit according to the manufacturer's instructions. After treatment, cells were harvested, washed twice with cold PBS, and resuspended in binding buffer. Annexin V-FITC and propidium iodide (PI) were added sequentially, and the cells were incubated in the dark for 15 minutes at room temperature. Flow cytometry analysis was performed using a flow cytometer (BD FACSCanto II), and data were analyzed using FlowJo software (version 10.0). Cells in the early apoptotic stage were defined as Annexin V-positive and PI-negative, while cells in the late apoptotic stage were defined as both Annexin V-positive and PI-positive. The total apoptosis rate was calculated as the sum of early and late apoptosis percentages.

2.9 Cell Cycle Analysis

Cell cycle distribution was analyzed by flow cytometry using propidium iodide (PI) staining. After treatment, cells were harvested, fixed in 70% ice-cold ethanol overnight at 4°C, and then treated with RNase A at 37°C for 30 minutes. PI was added to a final concentration of 50 micrograms per milliliter,

and the cells were incubated in the dark for 30 minutes at room temperature. Flow cytometry analysis was performed, and the proportions of cells in the sub-G1, G0/G1, S, and G2/M phases were determined using ModFit LT software.

2.10 Transwell Invasion Assay

Cell invasion capacity was evaluated using Transwell chambers pre-coated with Matrigel. After treatment, cells were seeded into the upper chamber in serum-free medium, while the lower chamber was filled with medium containing 10% FBS as a chemoattractant. After incubation for 24 hours, non-invading cells on the upper surface of the membrane were removed with a cotton swab. The invading cells on the lower surface were fixed with 4% paraformaldehyde and stained with crystal violet. The number of invaded cells was counted under a light microscope in five randomly selected fields per chamber, and the average number was calculated for statistical analysis.

2.11 Immunofluorescence Staining

Immunofluorescence staining was performed to evaluate the expression and localization of mitotic markers in colon cancer cells. Cells were seeded on coverslips in 24-well plates and treated according to the experimental groups. After treatment, cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.3% Triton X-100 for 10 minutes, and blocked with 5% BSA for 1 hour at room temperature. Primary antibodies against phospho-Histone H3 (Ser10, 1:500, Cell Signaling Technology, #9701) as the mitotic marker were applied and incubated overnight at 4°C. After washing with PBS, fluorescent secondary antibodies were applied and incubated for 1 hour at room temperature in the dark. Nuclei were counterstained with DAPI for 5 minutes. Fluorescence images were captured using a confocal laser scanning microscope, and the mitotic index was calculated as the percentage of mitotic cells among total DAPI-positive cells.

2.12 Statistical Analysis

All experiments were performed in triplicate, and data are presented as mean $\pm$ standard deviation (SD). Statistical comparisons between groups were performed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. A P-value < 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism software (version 9.0) or R software (version 4.3.0).

3. Results

3.1 Identification of Apocynin Target Genes and COAD DEGs

Based on the TCGA-COAD dataset, a total of 3,273 differentially expressed genes (DEGs) were identified using the criteria of $|\log_2\text{FoldChange}| > 1.5$ and adjusted P-value < 0.05 . The volcano plot (Fig. 1A) displays the distribution of DEGs, with upregulated genes shown in red, downregulated genes in blue,

and non-significant genes in gray. KLK6 was identified as the most significantly upregulated gene (highest $\log_2FC$ and $-\log_{10}P$ value), along with other notably upregulated genes including NOTUM, ZIC5, MAGEA3, and MAGEA6. Significantly downregulated genes included AQP8, OTOP2, CA1, MS4A10, and G6PC. The heatmap (Fig. 1B) further illustrated the expression patterns of representative DEGs between tumor tissues (red) and normal tissues (blue). ZIC5, KLK6, NOTUM, MAGEA3, and MAGEA6 were markedly overexpressed in tumor tissues, while AQP8, CA1, OTOP2, G6PC, and MS4A10 were significantly downregulated, demonstrating distinct expression profiles between tumor and normal tissues. Venn diagram analysis (Fig. 1C) revealed that 58 common genes (accounting for 1.6% of the total) were shared between the 3,273 DEGs and the 303 Apocynin target genes retrieved from the GeneCards database. These 58 intersection genes were defined as the potential core targets of Apocynin against colon cancer and were subjected to subsequent PPI network construction and pathway enrichment analysis.

3.2 PPI Network Construction and Hub Gene Identification

The 58 intersection target genes were imported into the STRING database to construct a PPI network (Fig. 2A), which revealed extensive protein-protein interactions among the target genes with a high network connectivity. Using the CytoHubba plugin in Cytoscape software, the top 10 genes were screened by the MCC and EPC algorithms, respectively (Fig. 2B). The top 10 genes identified by the MCC algorithm were CCND1, CXCL8, SERPINE1, SPP1, CXCL12, ALB, TIMP1, COL1A1, MMP13, and MMP1. The top 10 genes identified by the EPC algorithm were SERPINE1, CCND1, CXCL8, SPP1, HMOX1, CXCL12, TIMP1, ALB, AGT, and LEP. Node colors ranging from red to yellow represent rankings from high to low. Venn diagram analysis (Fig. 2C) revealed that 7 common hub genes (accounting for 53.8%) were shared between the two algorithms, namely CCND1, CXCL8, SERPINE1, SPP1, CXCL12, ALB, and TIMP1. Notably, CCND1 (Cyclin D1) is a key downstream effector of the PI3K/Akt pathway that regulates the G1/S cell cycle transition, while CXCL8 and SERPINE1 are closely associated with the tumor microenvironment and cell proliferation, further supporting the hypothesis that Apocynin may exert anti-colon cancer effects through the PI3K/Akt pathway.

3.3 GO and KEGG Enrichment Analysis

To further elucidate the biological functions and signaling pathways associated with the core target genes, KEGG pathway enrichment analysis and GO functional annotation were performed on the intersection target genes. The Sankey diagram (Fig. 3A) intuitively displayed the correspondence between the 7 hub genes (CCND1, CXCL12, CXCL8, SERPINE1, SPP1, and TIMP1) and the enriched KEGG pathways. CCND1 was involved in the greatest number of pathways, including the PI3K-Akt signaling pathway, p53 signaling pathway, Hippo signaling pathway, and Hedgehog signaling pathway, further confirming its importance as a core node. The KEGG pathway enrichment bubble plot (Fig. 3B) revealed that the intersection genes were significantly enriched in multiple tumor-related pathways, with the PI3K-Akt signaling pathway, p53 signaling pathway, NF- κ B signaling pathway, and HIF-1 signaling pathway all showing significant enrichment ($P_{\text{adjust}} < 0.05$). Additionally, focal adhesion, cellular senescence,

chemokine signaling pathway, cytokine-cytokine receptor interaction, and multiple infection-related pathways were also significantly enriched. GO functional annotation results (Fig. 3C) demonstrated the following: in terms of Biological Process (BP), the intersection genes were primarily enriched in positive regulation of leukocyte chemotaxis, regulation of leukocyte migration, response to cytokine, positive regulation of proteolysis, and platelet alpha granule regulation; in terms of Cellular Component (CC), enrichment was mainly observed in the endoplasmic reticulum lumen, secretory granule lumen, and cytoplasmic vesicle lumen; in terms of Molecular Function (MF), the genes were primarily enriched in cytokine activity, CXCR chemokine receptor binding, chemokine receptor binding, and protease binding. These results collectively indicate that the core target genes of Apocynin are mainly involved in biological processes including cell proliferation, apoptosis, cell cycle regulation, and immune-inflammatory responses, with the PI3K-Akt signaling pathway serving as one of the central pathways mediating its anti-colon cancer effects.

3.4 Molecular Docking Validation

Molecular docking was performed to validate the binding interactions between Apocynin and the seven hub gene-encoded proteins. The results demonstrated that Apocynin exhibited favorable binding affinities with all seven target proteins, with binding energies ranging from -4.2 to -6.8 kcal/mol (Table 1). Among these, CCND1 showed the strongest binding affinity with Apocynin (binding energy: -6.8 kcal/mol), followed by TIMP1 (-5.9 kcal/mol) and SERPINE1 (-5.6 kcal/mol). The binding energy values below -5.0 kcal/mol generally indicate strong and stable binding interactions. The docking results confirmed that Apocynin can effectively bind to the active sites of these hub gene-encoded proteins, particularly CCND1, forming hydrogen bonds and hydrophobic interactions that stabilize the drug-protein complexes. These computational findings provide structural evidence supporting the multi-target mechanism of Apocynin against colon cancer and are consistent with the network pharmacology predictions.

Table 1
Molecular docking results of Apocynin with hub gene-encoded proteins.

Hub Gene	Protein Name	PDB ID	Binding Energy (kcal/mol)
ALB	Albumin	1A06	-4.2
CCND1	Cyclin D1	2W96	-6.8
CXCL12	SDF-1	2KEG	-4.8
CXCL8	Interleukin-8	5D14	-5.1
SERPINE1	PAI-1	4AQH	-5.6
SPP1	Osteopontin	3BXD	-4.5
TIMP1	TIMP-1	1UEA	-5.9
Note: Binding energy < -5.0 kcal/mol indicates strong binding affinity. PDB ID refers to the Protein Data Bank identifier for each crystal structure used in docking.			

3.5 Survival Analysis of Hub Genes

Kaplan-Meier survival analysis was performed to evaluate the prognostic significance of the seven hub genes in COAD patients from the TCGA dataset. Patients were stratified into high-expression and low-expression groups based on the median expression level of each gene. The results revealed that CXCL8 was significantly associated with overall survival ($P = 0.00048$), with patients in the CXCL8 high-expression group demonstrating significantly poorer survival compared with those in the low-expression group. The survival difference for SERPINE1 approached statistical significance ($P = 0.057$). The remaining hub genes (ALB, CCND1, CXCL12, SPP1, and TIMP1) did not show statistically significant survival differences between high- and low-expression groups ($P > 0.05$) (Fig. 4). These results indicate that CXCL8 is aberrantly overexpressed in colon cancer tissues (Fig. 4A) and is significantly associated with poor patient prognosis (Fig. 4B), suggesting its potential role as a prognostic biomarker and therapeutic target in colon cancer.

3.6 Apocynin Promotes Apoptosis in Colon Cancer Cells

To validate the predicted anti-tumor effects of Apocynin, flow cytometry with Annexin V-FITC/PI double staining was performed to assess apoptosis in colon cancer cells across the four experimental groups (Control, HT, APO, and HT + APO). As shown in Fig. 5A-B, the total apoptosis rate was significantly increased in the APO group compared with the Control group, confirming that Apocynin effectively induces apoptosis in colon cancer cells. The HT group also showed a moderate increase in apoptosis compared with the Control group. Notably, the HT + APO combination group exhibited the highest apoptosis rate among all groups, which was significantly greater than that of either the HT or APO alone groups, indicating a synergistic pro-apoptotic effect of the combination treatment. These results demonstrate that Apocynin promotes colon cancer cell apoptosis and that its combination with hyperthermia enhances this effect in a synergistic manner.

3.7 Apocynin Induces Cell Cycle Arrest in Colon Cancer Cells

Cell cycle distribution was analyzed by flow cytometry using PI staining to determine whether Apocynin affects cell cycle progression in colon cancer cells. As shown in Fig. 6, the APO group exhibited a significant increase in the proportion of cells in the G0/G1 phase and a corresponding decrease in the S phase compared with the Control group, indicating that Apocynin induces G0/G1 cell cycle arrest. The HT group also showed a moderate G0/G1 arrest. The HT + APO combination group demonstrated the most pronounced cell cycle arrest, with the highest proportion of cells in the G0/G1 phase and the lowest proportion in the S and G2/M phases. Additionally, the sub-G1 population, which represents apoptotic cells with fragmented DNA, was markedly increased in the HT + APO group compared with the other groups, further corroborating the apoptosis-promoting effect of the combination treatment. These findings are consistent with the network pharmacology prediction that CCND1, a key regulator of G1/S transition, is a major target of Apocynin, and suggest that Apocynin inhibits colon cancer cell proliferation at least in part through CCND1-mediated cell cycle arrest.

3.8 Apocynin Inhibits Invasion of Colon Cancer Cells

The Transwell invasion assay was performed to evaluate the effect of Apocynin on the invasive capacity of colon cancer cells. As shown in Fig. 7A-B, the number of invaded cells was significantly reduced in the APO group compared with the Control group, indicating that Apocynin effectively suppresses the invasive ability of colon cancer cells. The HT group also showed a moderate reduction in cell invasion. Importantly, the HT + APO combination group exhibited the lowest number of invaded cells among all groups, demonstrating the most potent anti-invasive effect. The synergistic inhibition of invasion by the HT + APO combination was statistically significant compared with either treatment alone. These results are consistent with the enrichment of cell migration-related pathways in the GO analysis and suggest that Apocynin may inhibit colon cancer metastasis by targeting key regulators of cell invasion, including SERPINE1 and TIMP1, which are involved in extracellular matrix remodeling and tumor cell invasion.

3.9 Apocynin Reduces Mitotic Index in Colon Cancer Cells

Immunofluorescence staining was performed to assess the effect of Apocynin on the mitotic activity of colon cancer cells. Cells were stained with antibodies against a mitotic marker, DAPI for nuclear visualization, and the merged images were analyzed to determine the mitotic index. As shown in Fig. 8A-B, the Control group exhibited strong mitotic marker expression, indicating active cell proliferation. Both the APO and HT groups showed moderate reductions in mitotic marker expression compared with the Control group. The HT + APO combination group displayed the weakest mitotic marker expression and the lowest mitotic index among all groups, indicating the most significant suppression of cell proliferation. The quantitative analysis confirmed that the mitotic index was significantly decreased in the HT + APO group compared with the Control, HT, and APO groups. These findings are consistent with

the cell cycle analysis results and further support the anti-proliferative effect of Apocynin on colon cancer cells, particularly in combination with hyperthermia.

4. Discussion

This study systematically investigated the anti-COAD mechanisms of Apocynin through an integrated network pharmacology, molecular docking, and in vitro validation approach. We identified 58 overlapping targets between Apocynin and COAD, extracting seven hub genes (ALB, CCND1, CXCL12, CXCL8, SERPINE1, SPP1, TIMP1). KEGG enrichment predominantly highlighted the PI3K/Akt signaling pathway, a central regulator of cell survival and proliferation frequently dysregulated in colon cancer. Molecular docking demonstrated that Apocynin exhibits strong binding affinity to these hub proteins, particularly CCND1 (-6.8 kcal/mol). Experimentally, Apocynin promoted apoptosis, induced G0/G1 arrest, inhibited invasion, and reduced the mitotic index, with hyperthermia (HT) combination yielding synergistic anti-tumor effects. Among the hub genes, CCND1 and CXCL8 are of paramount importance. CCND1 encodes cyclin D1, a crucial G1/S checkpoint regulator commonly overexpressed in colon cancer²⁰. The strong Apocynin-CCND1 binding affinity computationally predicted in our docking analysis aligns with the observed G0/G1 cell cycle arrest in vitro, suggesting Apocynin directly targets cyclin D1 to impede cell cycle progression. Concurrently, CXCL8 emerged as a significant adverse prognostic biomarker ($P = 0.00048$). As a pro-inflammatory chemokine, CXCL8 fosters tumor angiogenesis, invasion, and immune evasion²¹. Apocynin's predicted interaction with CXCL8, alongside the enrichment of IL-17 and NF- κ B pathways, implies its capacity to suppress the pro-tumorigenic microenvironment. Notably, ALB (albumin) is primarily a serum transport protein rather than an intracellular signaling target; its inclusion likely reflects systemic metabolic alterations associated with cancer rather than a direct druggable target in colon cancer cells. We therefore focused mechanistic interpretation on the remaining six hub genes with established roles in colon cancer cell signaling. A critical finding is the synergistic efficacy of the Apocynin-HT combination. HT likely enhances Apocynin cellular uptake via increased membrane permeability and sensitizes cancer cells to PI3K/Akt pathway inhibition through thermal protein denaturation, thereby amplifying apoptosis and cell cycle arrest. The synergistic reduction in mitotic index achieved $P < 0.05$ significance, whereas apoptosis, cell cycle arrest, and invasion inhibition reached $P < 0.01$, suggesting the combination effect on proliferation markers may exhibit a slightly delayed onset compared with immediate cytotoxic responses.

Several limitations should be acknowledged. First, while network pharmacology strongly implicates the PI3K/Akt pathway, our in vitro experiments confirmed phenotypic changes without directly measuring PI3K/Akt protein activity (e.g., p-PI3K, p-Akt by Western blot). Second, direct experimental validation of Apocynin-mediated CXCL8 modulation (e.g., ELISA, qPCR) was not performed. Third, the reliance on in vitro models necessitates in vivo xenograft validation. Fourth, ALB's inclusion as a hub gene likely reflects systemic metabolic associations rather than a direct intracellular target.

5. Conclusion

In conclusion, this study systematically investigated the anti-COAD mechanisms of Apocynin through an integrated approach combining network pharmacology, molecular docking, and in vitro experimental validation. Our bioinformatics analyses suggest that Apocynin may exert anti-tumor effects through modulation of the PI3K/Akt signaling pathway and regulation of key hub genes including CCND1 and CXCL8, with molecular docking confirming strong binding affinity between Apocynin and CCND1. Experimental validation demonstrated that Apocynin promotes apoptosis, induces G0/G1 cell cycle arrest, inhibits cell invasion, and reduces the mitotic index in colon cancer cells. Importantly, the combination of Apocynin with hyperthermia exhibited synergistic anti-tumor effects, providing a rationale for the development of novel combination therapeutic strategies. CXCL8 was identified as a significant adverse prognostic biomarker for COAD, supporting its potential clinical utility for patient stratification. Future studies should prioritize direct PI3K/Akt pathway validation using Western blot and rescue experiments, in vivo xenograft validation, and optimization of the Apocynin-hyperthermia combination regimen for clinical translation.

Declarations

Ethics approval and consent to participate

Not applicable. This study utilized publicly available data from TCGA and GeneCards databases, and in vitro cell line experiments that do not require ethics approval.

Consent for publication

Not applicable.

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Author Contribution

Huifang Si and Baohong Xu conceived and designed the study. Huifang Si and Jingnan Yang performed the bioinformatics analysis and molecular docking. Huifang Si and Chunsheng Ding conducted the in vitro experiments. Huifang Si drafted the manuscript. Baohong Xu revised the manuscript critically. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Public data sources include TCGA (<https://portal.gdc.cancer.gov/>), GeneCards (<https://www.genecards.org/>), STRING (<https://string-db.org/>), PubChem (<https://pubchem.ncbi.nlm.nih.gov/>), and PDB (<https://www.rcsb.org/>) .

Competing interests

The authors declare that they have no competing interests.

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Figures

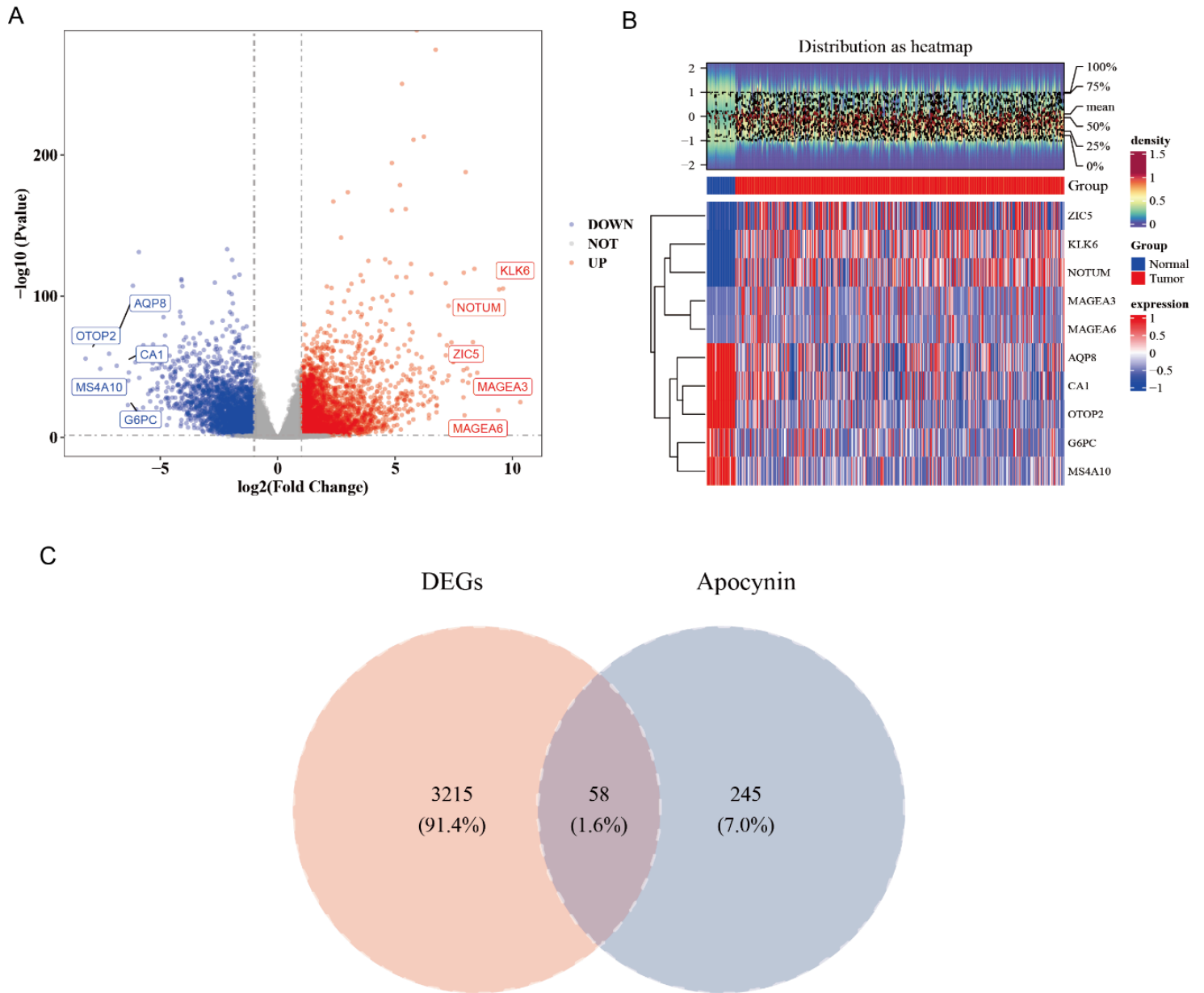


Figure 1

Identification of Apocynin target genes and COAD DEGs. (A) Volcano plot of differentially expressed genes in the TCGA-COAD dataset. Red dots represent upregulated genes, green dots represent downregulated genes, and black dots represent non-significant genes ($|\log_2\text{FoldChange}| > 1.5$, adjusted $P < 0.05$). (B) Heatmap showing the expression distribution of differentially expressed genes between normal and tumor tissues. (C) Venn diagram showing the overlap between Apocynin-related target genes and COAD DEGs, yielding common target genes.

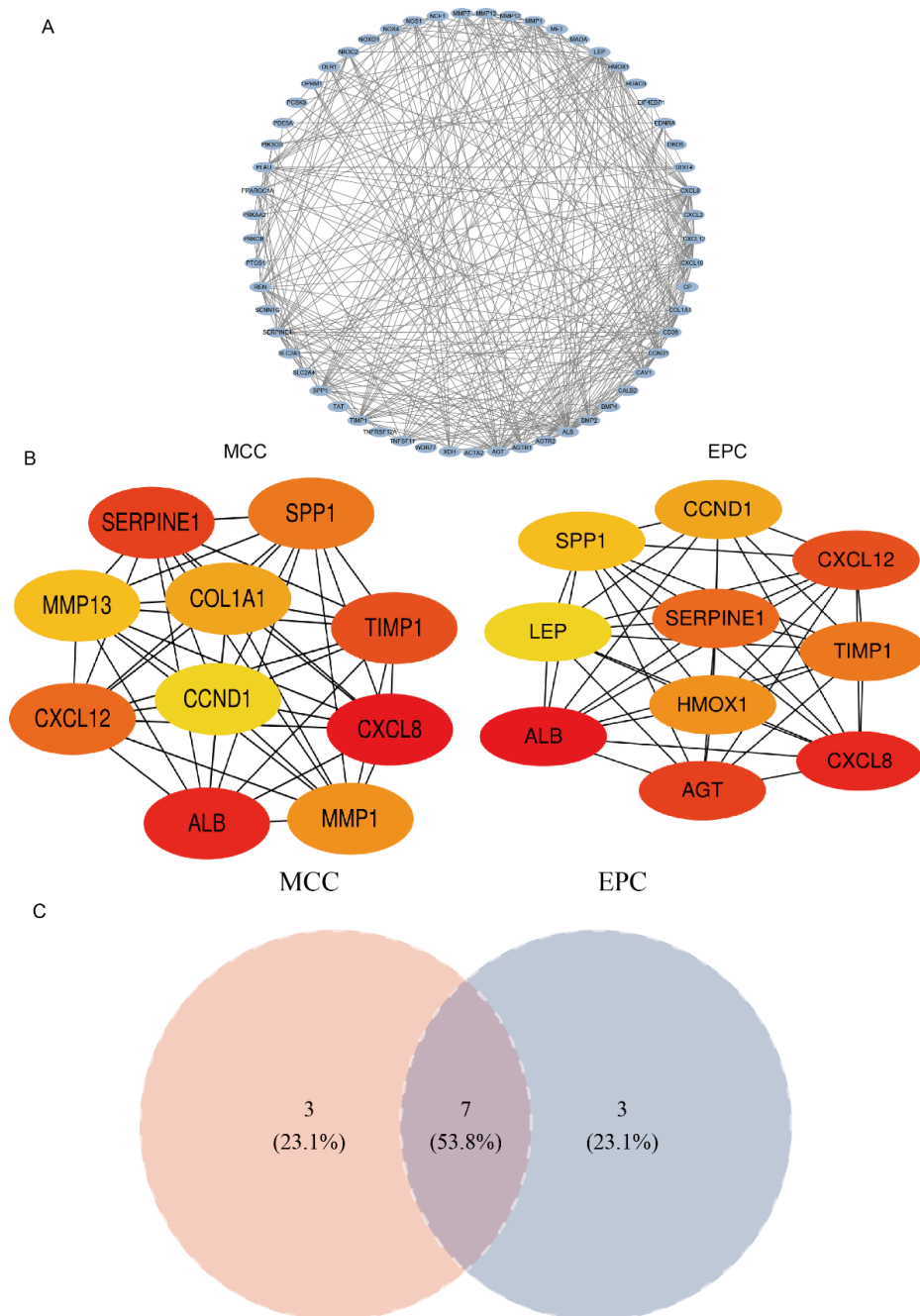


Figure 2

PPI network and hub gene identification. (A) Protein-protein interaction network of 58 overlapping genes constructed using the STRING database. (B) Hub genes identified by CytoHubba using the MCC and EPC algorithms. The top 7 hub genes (ALB, CCND1, CXCL12, CXCL8, SERPINE1, SPP1, and TIMP1) are highlighted in the network. (C) Venn diagram showing the overlap of hub genes identified by the MCC and EPC algorithms, with 7 common hub genes shared between both methods.

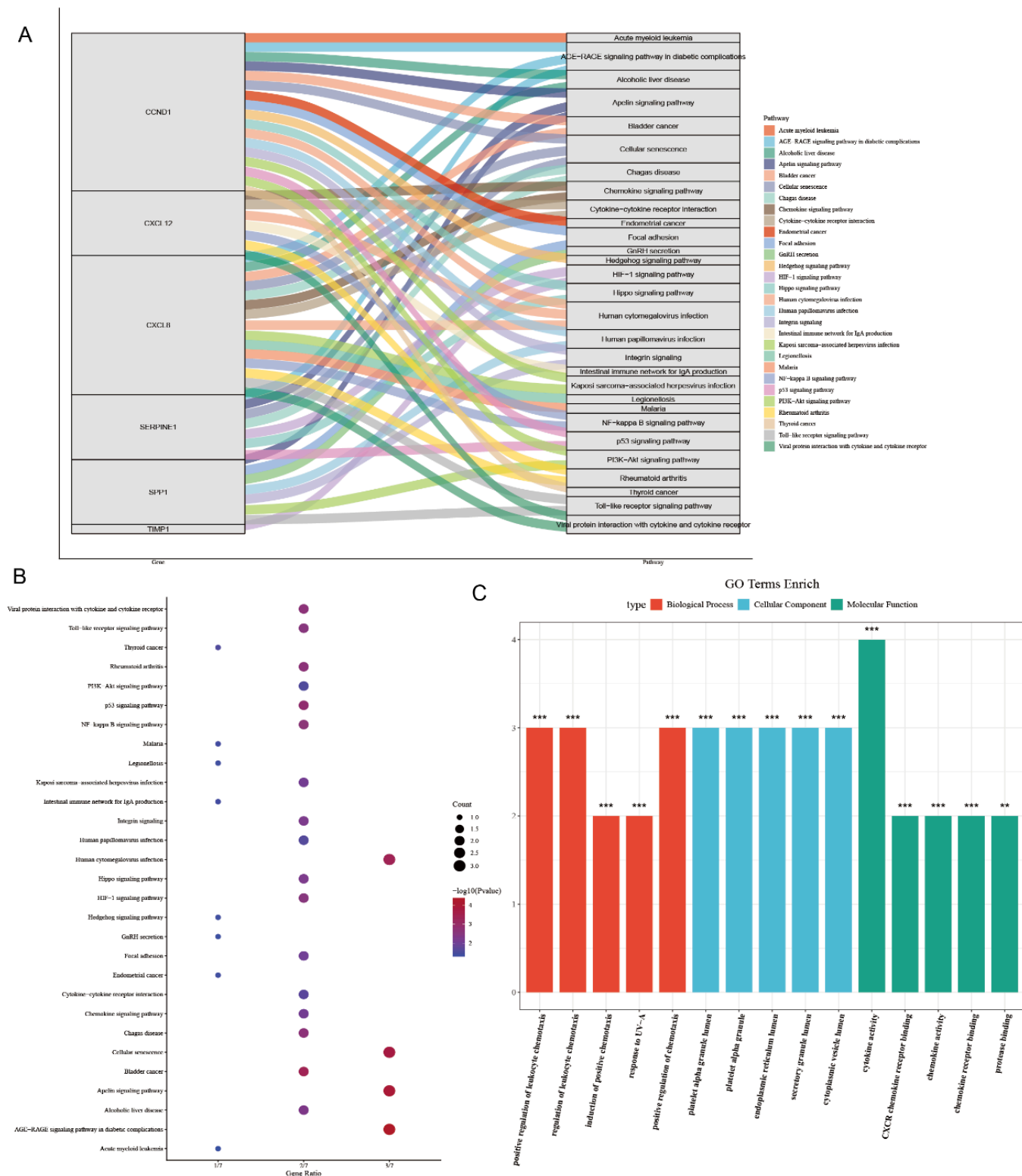


Figure 3

KEGG pathway enrichment analysis and GO functional annotation of the intersection target genes. (A) Sankey diagram illustrating the correspondence between hub genes (left) and enriched KEGG pathways (right). The width of connecting lines reflects the strength of association between genes and pathways. (B) KEGG pathway enrichment bubble plot. The x-axis represents Gene Ratio, and the y-axis represents pathway names. Bubble size indicates the number of enriched genes (Count), and color represents $-\log_{10}$

(P value), with deeper colors indicating more significant enrichment. (C) GO functional annotation bar chart, comprising three categories: Biological Process (red), Cellular Component (blue), and Molecular Function (green). The y-axis represents $-\log_{10}$ (P value). $P < 0.001$.

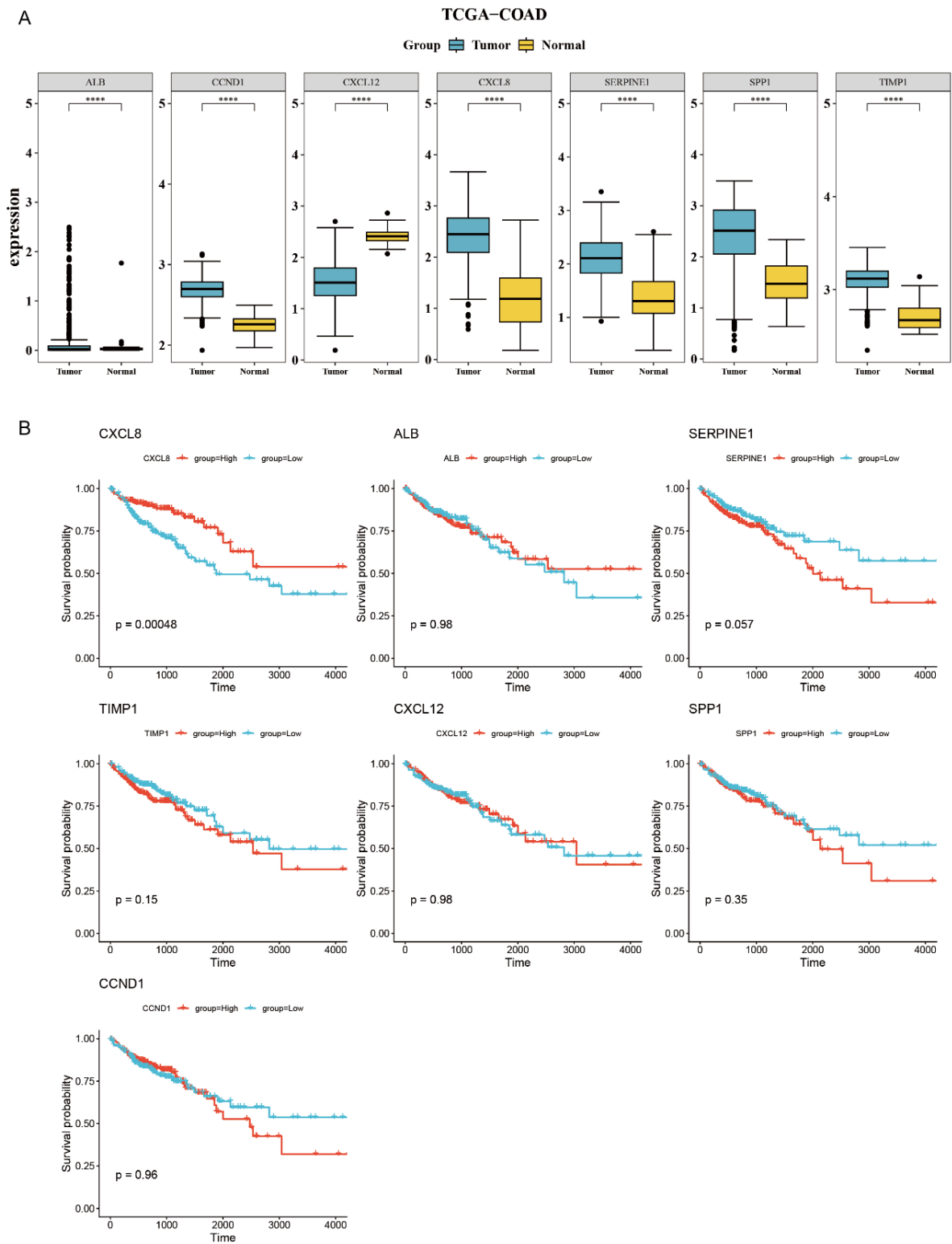


Figure 4

Differential expression and survival analysis of hub genes in colon cancer. (A) Box plots showing the expression levels of 7 hub genes (ALB, CCND1, CXCL12, CXCL8, SERPINE1, SPP1, and TIMP1) in tumor tissues (Tumor, blue) and normal tissues (Normal, yellow) based on the TCGA-COAD dataset. $P < 0.0001$. (B) Kaplan-Meier overall survival curves for the 7 hub genes. Patients were stratified into high-expression (High, red) and low-expression (Low, blue) groups based on the median expression level of each gene. P values were calculated by the log-rank test.

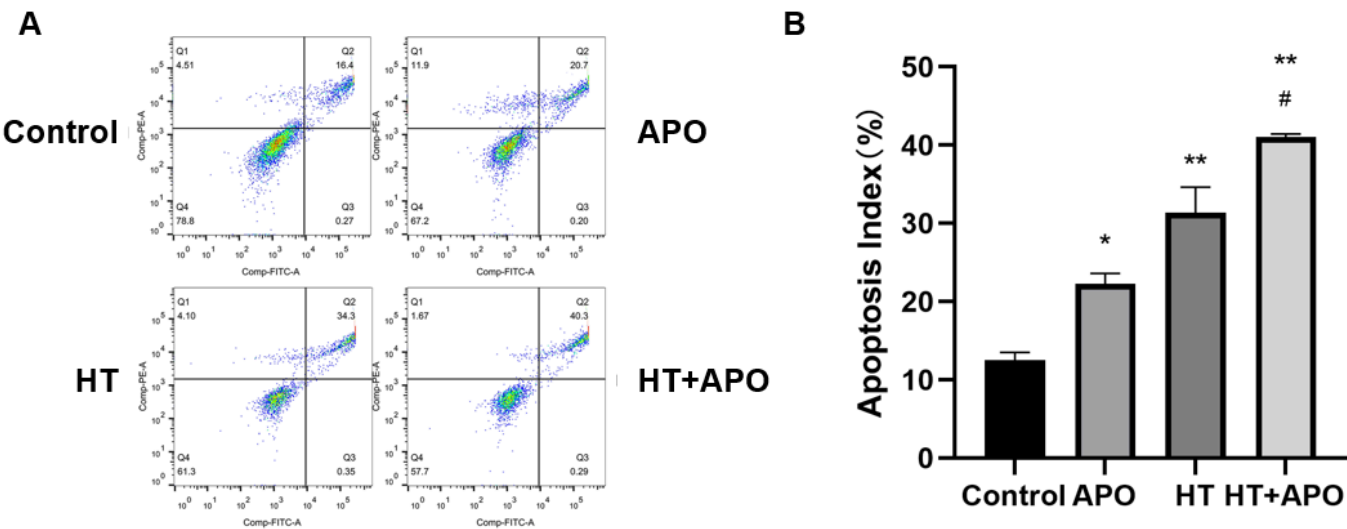


Figure 5

Apocynin promotes apoptosis in colon cancer cells. (A) Flow cytometry analysis of apoptosis using Annexin V-FITC/PI double staining in Control, HT, APO, and HT+APO groups. The upper right quadrant (Q2, Annexin V+/PI+) represents late apoptotic cells, the lower right quadrant (Q3, Annexin V+/PI-) represents early apoptotic cells, and the lower left quadrant (Q4, Annexin V-/PI-) represents viable cells. (B) Quantitative analysis of total apoptosis rate (Q2 + Q3) across the four groups. $P < 0.01$ vs. Control; ## $P < 0.01$ vs. HT or APO alone.

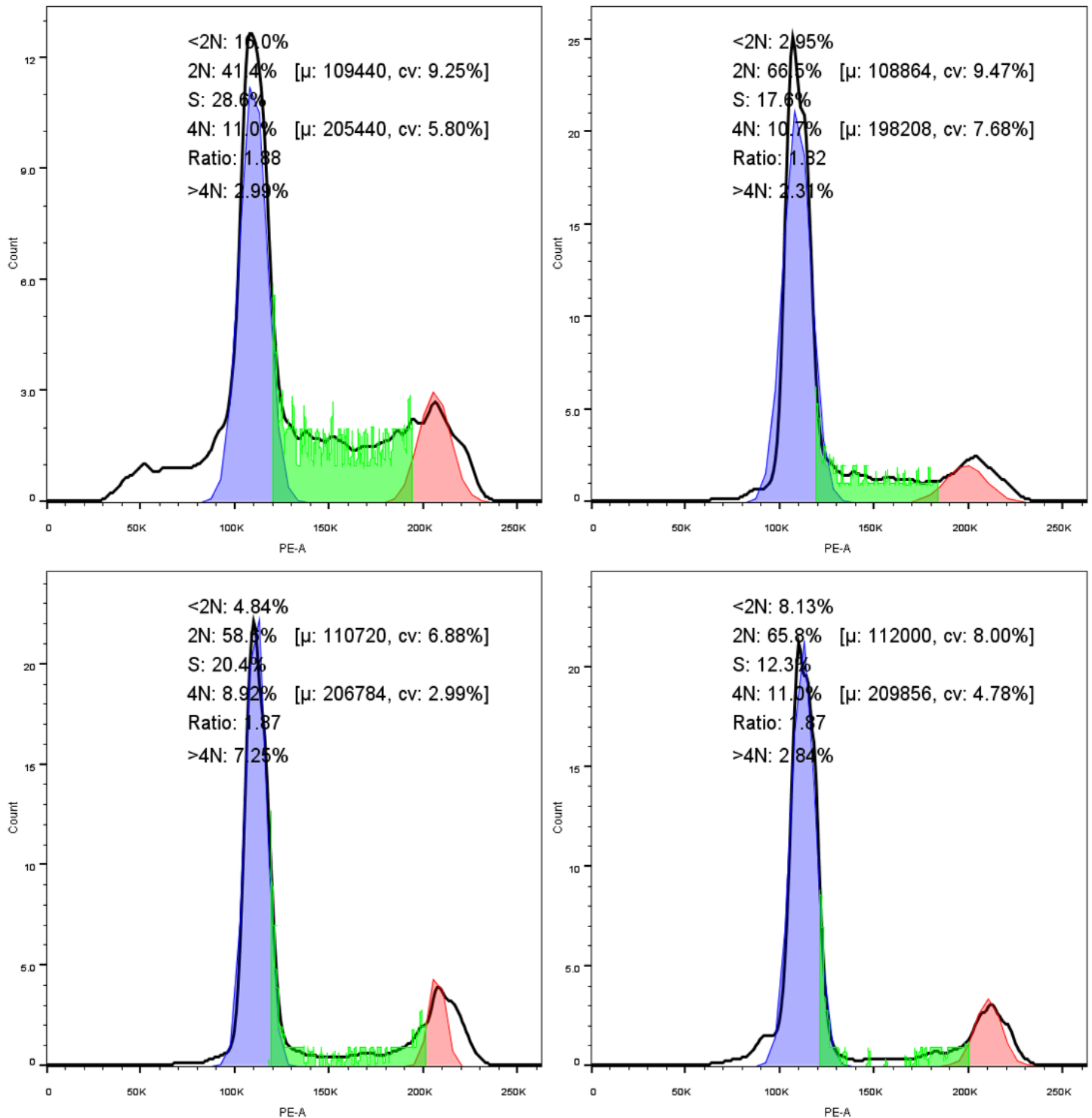


Figure 6

Apocynin induces G0/G1 cell cycle arrest in colon cancer cells. Flow cytometry analysis of cell cycle distribution in Control, APO, HT, and HT+APO groups using PI staining. The percentages of cells in sub-G1, G0/G1, S, and G2/M phases are indicated for each group. Quantitative analysis of cell cycle distribution across the four groups. The HT+APO group shows the most pronounced G0/G1 arrest and the highest sub-G1 population. $P < 0.01$ vs. Control; $##P < 0.01$ vs. HT or APO alone.

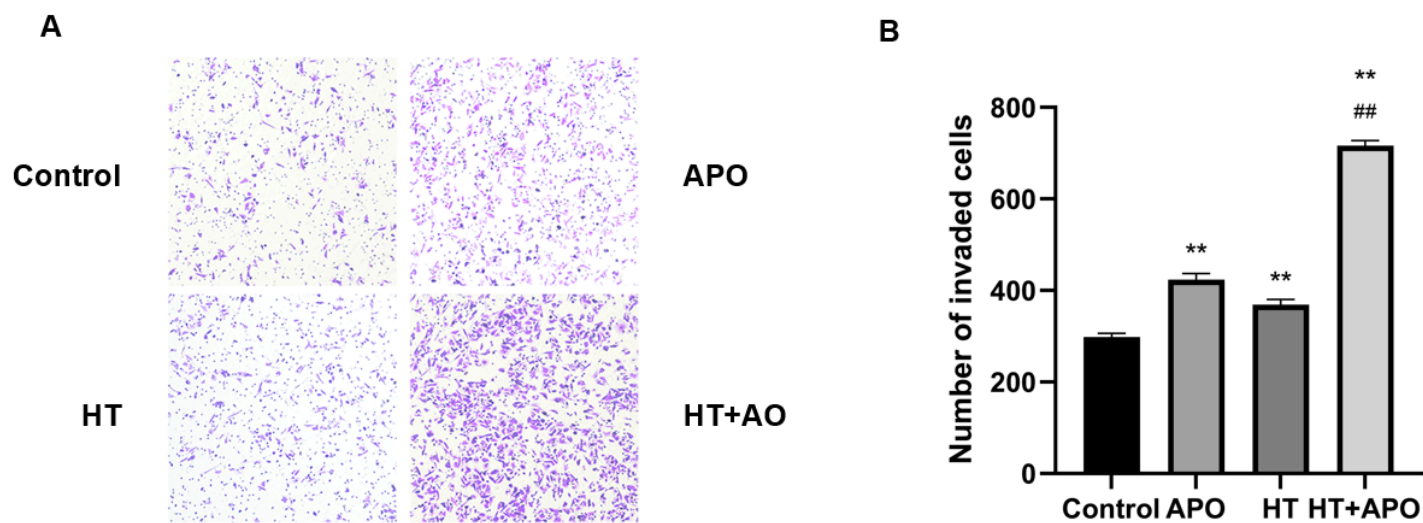


Figure 7

Apocynin inhibits invasion of colon cancer cells. (A) Transwell invasion assay showing crystal violet-stained invaded cells in Control, HT, APO, and HT+APO groups. Scale bar = 100 μ m. (B) Quantitative analysis of the number of invaded cells across the four groups. Data are presented as mean $\pm$ SD from three independent experiments. $P < 0.01$ vs. Control; ## $P < 0.01$ vs. HT or APO alone.

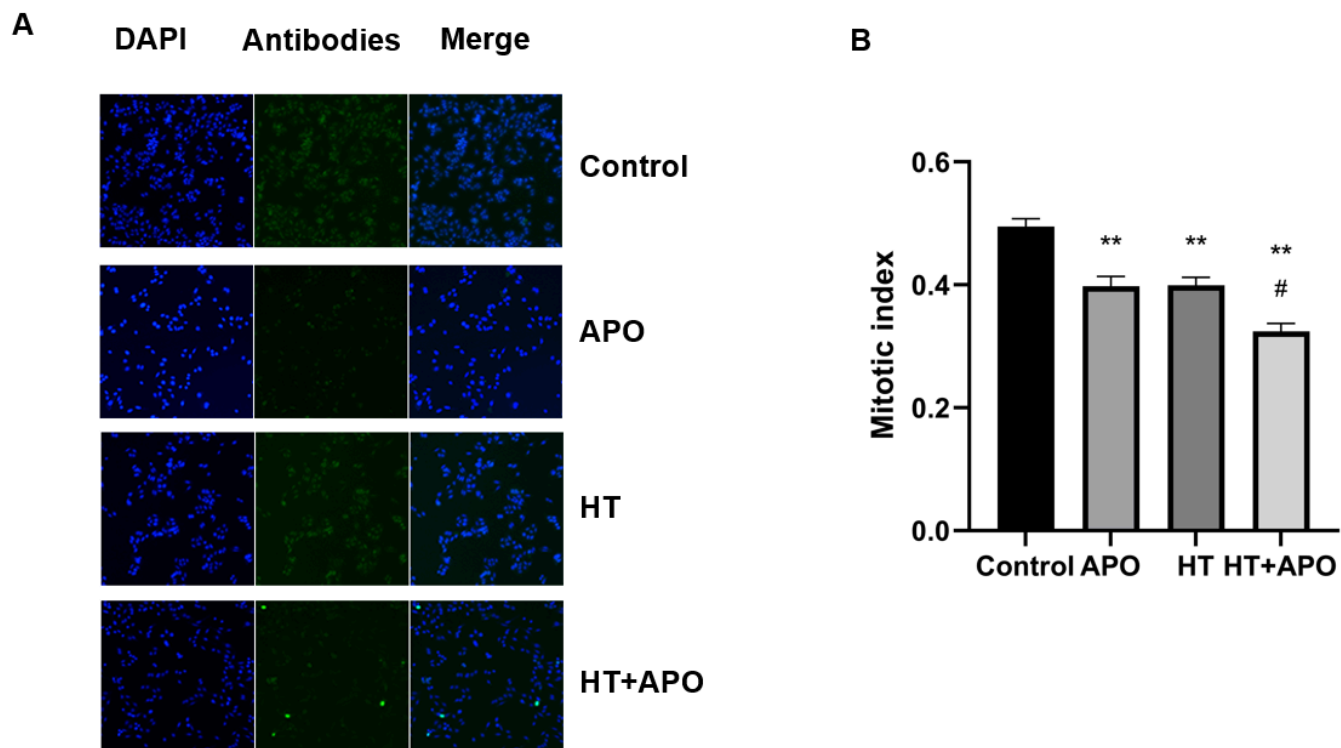


Figure 8

Apocynin reduces mitotic index in colon cancer cells. (A) Immunofluorescence staining showing DAPI (blue), mitotic marker antibody (green), and merged images in Control, APO, HT, and HT+APO groups. Scale bar = 50 μ m. (B) Quantitative analysis of mitotic index across the four groups. Data are presented as mean $\pm$ SD from three independent experiments. $P < 0.01$ vs. Control; $\#P < 0.05$ vs. HT or APO alone.