Alisol A attenuates malignant phenotypes of colorectal cancer cells by inactivating PI3K/Akt signaling

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Abstract. Despite the advancement in the diagnosis and therapeutic strategies for colorectal cancer, the outcomes of patients with colorectal cancer remain unsatisfactory. Alisol A is a natural constituent of Alismatis rhizoma (zexie) and has demonstrated anti-cancer properties; however, the function of Alisol A in colorectal cancer is still unknown. In the present study, the effect of Alisol A on colorectal cancer progression was investigated. MTT and colony formation assays showed that treatment with Alisol A repressed colorectal cancer cell proliferation in a dose-dependent manner. Similarly, western blot analysis demonstrated that Alisol A upregulated E-cadherin protein expression levels, but downregulated N-cadherin and Vimentin protein expression levels in colorectal cancer cells. In addition, the number of cells in G_0/G_1 phase was enhanced, while that of S phase was reduced in Alisol A-treated colorectal cancer cells. Apoptosis and pyroptosis of colorectal cancer cells were stimulated following treatment with Alisol A. Alisol A suppressed the migration ability of colorectal cancer cells in a dose-dependent manner. Moreover, Alisol A increased the chemotherapeutic sensitivity of colorectal cancer cells to cisplatin. Mechanically, western blot analysis confirmed that Alisol A repressed the phosphorylation levels of PI3K, Akt and mTOR in colorectal cancer cells. The Akt activator, SC79 reversed the effect of Alisol A on colorectal cancer cell proliferation and apoptosis. In conclusion, Alisol A induced an inhibitory effect on colorectal cancer progression by inactivating PI3K/Akt signaling.

Introduction

Colorectal cancer is the primary cause of tumor-associated mortality and morbidity worldwide (1-3). Notwithstanding the advantages of early high-quality detection, screening, diagnosis, new chemotherapies, and surgery for enhanced therapy and prognosis in patients with metastatic and advanced colorectal cancer, the 5-year survival rate of these colorectal cancer cases is still <10% (4,5). Even though these therapeutic strategies attenuate cancer-related growth and metastasis, they can result in severe toxic effects, affecting healthy tissues, and interfering with therapeutic progress and patient quality of life (6,7). Consequently, the investigation and development of practical and effective therapeutic agents, and strategies are urgently required to improve the clinical outcomes of patients with colorectal cancer.

Traditional Chinese natural compounds are a valuable source of tumor therapeutic candidates due to their practical effectiveness and low toxicity (8,9). Alismatis rhizoma (zexie) serves as the rhizome of Alisma Orientale, which is an oceanic herb belonging to the Alismataceae family and is broadly distributed in Japan, Korea, and China (10). It has been extensively adopted as a hypolipidemic agent and folk diuretic for >1,000 years in China and has been used to treat urinary tract infections, edema, hypertension, and dysuria (11). Modern research medicine has confirmed the anti-atherosclerotic, hypoglycemic, antihypertensive, diuretic and anti-cancer effects of Alismatis rhizoma (12). Alismatis rhizoma contains several active chemical constituents, including essential oils, diterpenes, sesquiterpenes, polysaccharides and triterpenoids (13). Alisol A serves as a tetracyclic protostane-type triterpenoid and a major component of Alismatis rhizoma (14). A study has identified the antitumor effect of Alisol A on breast cancer cells (15), but the function of Alisol A in colorectal cancer development remains unknown.

In the present study, the effect of Alisol A on proliferation, cell cycle, apoptosis, pyroptosis, migration and invasion was investigated. The results confirmed that Alisol A attenuated colorectal cancer by targeting PI3K/Akt signaling.

Materials and methods

Cell culture and cell treatment. Colorectal cancer cell lines (HCT-116 and HT-29) were obtained from the Cell Bank of the Chinese Academy of Sciences. The HCT-116 and HT-29 cells were cultured in RPMI-1640 medium with 10% FBS (both Biological Industries) and 1% penicillin/streptomycin (Gibco;

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Thermo Fisher Scientific, Inc.). Short tandem repeat confirmation of the HT-29 cell line (no. VCPO20210816006STR01) was conducted by Shanghai VivaCell Biosciences Ltd. Alisol A was obtained from the National Pharmaceutical Engineering Research Center and its chemical structure is shown in Fig. 1A. The Akt inhibitor, SC79, and cisplatin (CIS) were purchased from Sigma-Adrich (Merck KGaA). In rescue experiments, Alisol A and SC79 were simultaneously added to cells to validate the effect of PI3K/Akt pathway in colorectal cancer cells.

MTT assays. MTT assays were performed to analyze the effect of Alisol A at concentrations of 5, 10, 20, 40, 80, and 160 μM on the cytotoxicity of HCT-116 and HT-29 cells. Approximately, $5x10^3$/well cells were seeded into 96-well plates and cultured for 24 h at 37°C. Then, MTT was added to the cells for 4 h at 37°C. Following removal of MTT solution, DMSO (200 μl) was added to each well. The optical density (490 nm) was determined using a microplate reader (Bio-Rad Laboratories, Inc.).

Colony formation assays. A total of 500 HCT-116 and HT-29 cells were seeded into 6-well plates and cultured for 14 days. The cells were then fixed with 4% paraformaldehyde for 30 min at room temperature, stained with 1% crystal violet (Beyotime Institute of Biotechnology) for 20 min at room temperature, and washed three times with PBS. Finally, the numbers of colonies containing >500 cells were assessed using a light microscope (magnification, x100).

Apoptosis and cell cycle analysis. For cell apoptosis analysis, the HCT-116 and HT-29 cells ($2x10^5$ in each well) were resuspended in RNase (Sigma-Aldrich), then stained with PI and Annexin V-fluorescein isothiocyanate (Beyotime Institute of Biotechnology) for 20 min in the dark at 37°C. The samples were then analyzed with a FACScan flow cytometer (BD Biosciences), using CellQuest Pro software(version 5.1, BD Biosciences).

For the cell cycle analysis, the HCT-116 and HT-29 cells were collected, washed with PBS, and then fixed for 24 h with 70% ice-cold ethanol at 4°C. Subsequently, RNase (MilliporeSigma) was added to the cells and incubated for 30 min at 37°C. The cells were then stained with PI (Beyotime Institute of Biotechnology) for 30 min at 4°C in the dark. Finally, cell cycle distribution was then determined using FACSCalibur (BD Biosciences), using ModFit LT software (version 3.1, Verity Software House).

Western blot analysis. RIPA (Beijing Solarbio Science & Technology Co., Ltd.) was used to prepare the cell lysate from HCT-116 and HT-29 cells. An enhanced BCA protein assay kit (Beyotime Institute of Biotechnology) was used to quantify the protein concentration. Then, the protein samples (50 μg) were separated using 10% SDS-PAGE and transferred to PVDF membranes (0.45 μm; MilliporeSigma). The membrane was incubated with antibodies against anti-Bcl-2 (1:1,000, ab182858, Abcam), anti-Bax (1:1,000, ab32503, Abcam), anti-cleaved caspase 3 (1:500, ab32042, Abcam), anti-caspase 3 (1:1,000, ab32351, Abcam), anti-cleaved PARP (1:1,000, ab32064, Abcam), anti-PARP (1:1,000, ab191217, Abcam), anti-caspase 1 (1:1,000, #83383, Cell Signaling Technology, Inc.), anti-cleaved caspase 1 (1:500, #4199, Cell Signaling Technology, Inc.), anti-GSDMD (1:1,000, ab210070, Abcam), anti-GSDME (1:1,000, ab215191, Abcam), anti-E-cadherin (1:1,000, ab231303, Abcam), anti-N-cadherin (1:1,000, ab76011, Abcam), anti-Vimentin (1:1,000, ab20346, Abcam), anti-PI3K (1:1,000, #4255, Cell Signaling Technology, Inc.), anti-Akt (1:1,000, #4691, Cell Signaling Technology, Inc.), anti-mTOR (1:1,000, #2983, Cell Signaling Technology, Inc.), anti-p-PI3K (1:1,000, #13857, Cell Signaling Technology, Inc.), anti-p-Akt (1:1,000, #4060, Cell Signaling Technology, Inc.), anti-p-mTOR (1:1,000, #2971, Cell Signaling Technology, Inc.), and anti-GAPDH (1:1,000, ab125247, Abcam) (overnight at 4°C) after blocking with 5% skimmed milk (2 h at 37°C). The results were observed using an ECL kit (MilliporeSigma) after incubation with horseradish peroxidase-labeled secondary antibody (1:5,000, ab288151, Abcam) at room temperature for 2 h.

Lactate dehydrogenase (LDH) analysis. LDH levels were measured using a LDH cytotoxicity assay kit (BioVision, Inc.). Briefly, the cells were prepared using Triton X-100 (0.2%; MilliporeSigma) and treated with LDH reaction solution (100 μl; 30 min). The results were observed using a microplate reader (490 nm; Bio-Rad Laboratories, Inc.).

Wound healing assay. The HCT-116 and HT-29 cells ($3x10^5$ cells/well) were seeded into 24-well plates. The cells were cultured overnight at 37°C to 100% confluency, and wounds were created in the cell monolayer using a 10-μl plastic pipette tip. Subsequently, the cells were washed with PBS for three times, and then serum-free medium was added into the wells for continuous incubation. At 0 and 24 h after scratching, the images were captured with a light microscope (magnification, x100).

Evaluation of drug sensitivity. HCT-116 and HT-29 cells ($2x10^4$ cells/well) were added into 96-well plate. When the cells adhered to the wall, CIS at different concentration was added into the well. Subsequently, the cells were cultured in 5% CO_2 at 37°C for 24 h. Then, Alisol A was added into the cells. Finally, MTT assay and clone formation assay were performed to evaluate the effect of Alisol A on the chemotherapeutic sensitivity of colorectal cancer cells to CIS.

Bioinformatics. Signaling pathways involving Alisol A and colorectal cancer were obtained via Bioinformatics Analysis Tool for Molecular mechanism of Traditional Chinese Medicine (BATMAN; bionet.ncpsb.org.cn/batman-tcm/) and Comparative Toxicgenomics Database (CTD; ctdbase.org/). Then, the signaling pathways were intersected using Venn analysis (version 2.1, bioinfogp.cnb.csic.es/tools/venny/).

Statistical analysis. The experiments were performed three times independently and the results are presented as the mean ± SEM. The data were evaluated from multiple groups using one-way ANOVA with a Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.
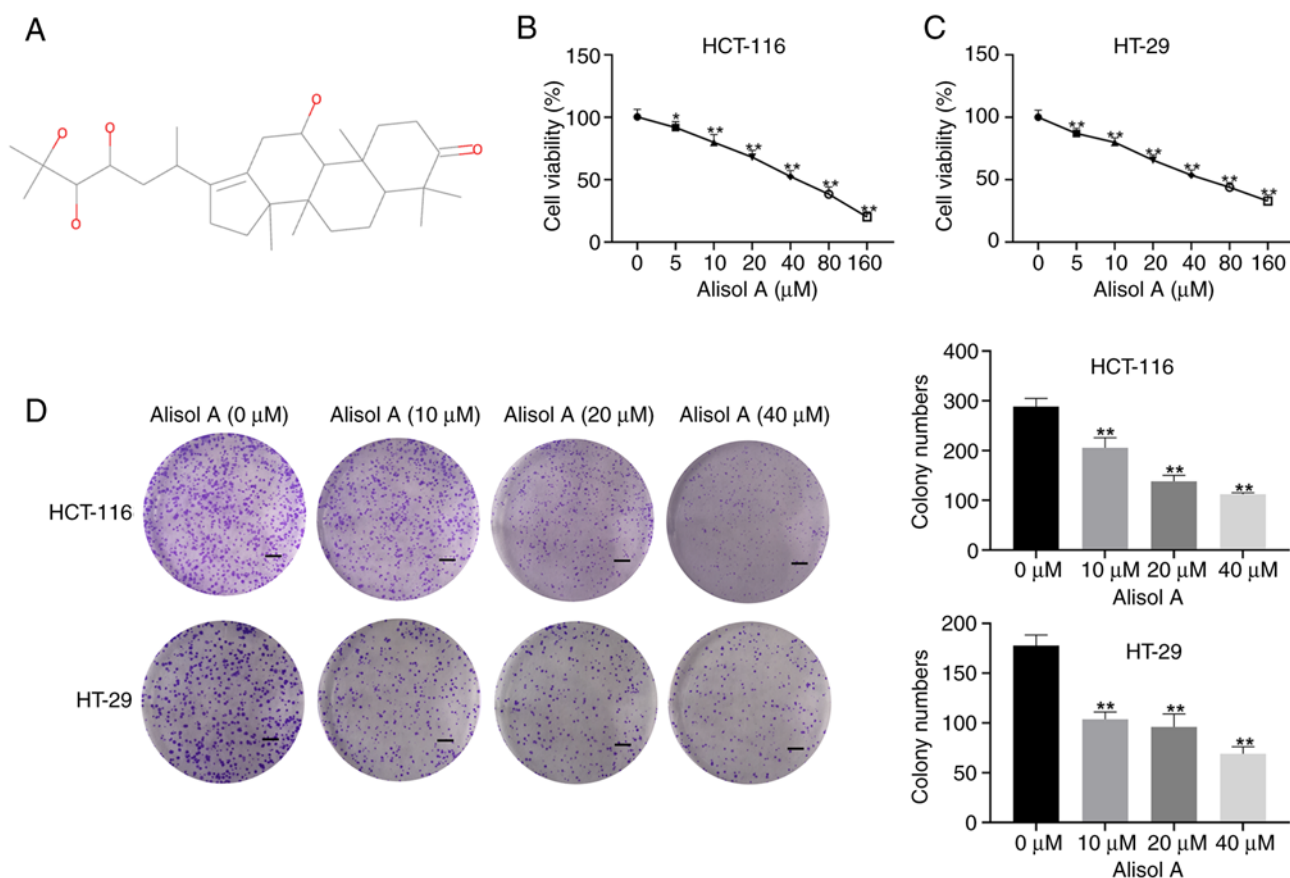
Figure 1. Alisol A suppresses colorectal cancer cell proliferation *in vitro*. (A) The chemical structure of Alisol A. MTT assay was performed in the (B) HCT-116 and (C) HT-2 cell line following treatment with different concentrations of Alisol A. (D) Colony formation assay was performed in the HCT-116 and HT-29 cell lines following treatment with different concentrations of Alisol A. The results are presented as the mean ± SEM. n=3. *P<0.05, **P<0.01 vs. Alisol A (0 μM).

Results

Alisol A represses colorectal cancer cell proliferation in vitro. The function of Alisol A in modulating colorectal cancer cell proliferation *in vitro* was assessed. MTT assays showed that treatment with Alisol A dose-dependently decreased cytotoxicity to HCT-116 and HT-29 cells at concentrations of 5, 10, 20, 40, 80, and 160 μM (Fig. 1B and C), and 10, 20 and 40 μM Alisol A were used for subsequent analysis. Similarly, the colony formation numbers of the HCT-116 and HT-29 cells were decreased by Alisol A (Fig. 1D and E), indicating that Alisol A represses colorectal cancer cell proliferation *in vitro*.

Alisol A induces cell cycle arrest in the colorectal cancer cells. Then, the potential role of Alisol A in the cell cycle of colorectal cancer cells was detected. For this purpose, the HCT-116 and HT-29 cells were treated with Alisol A at concentrations of 10, 20, and 40 μM and subjected to cell cycle analysis using flow cytometry. These data showed that the number of cells in G_0/G_1 phase was increased, while the number of cells in S phase was decreased in Alisol A-treated HCT-116 and HT-29 cells (Fig. 2A and B), suggesting that Alisol A induces cell cycle arrest in colorectal cancer cells.

Alisol A induces apoptosis and pyroptosis of colorectal cancer cells. Next, the effect of Alisol A on apoptosis and pyroptosis of colorectal cancer cells was evaluated. The

results demonstrated that HCT-116 and HT-29 cell apoptosis was stimulated following treatment with Alisol A (Fig. 3A and B). The expression levels of apoptosis-related proteins were detected and it was found that Alisol A inhibited Bcl-2 protein expression level, but increased Bax, cleaved caspase3, and cleaved PARP protein expression levels in the HCT-116 and HT-29 cells (Fig. 3C and D). In addition, the levels of LDH were enhanced in Alisol A-treated HCT-116 and HT-29 cells (Fig. 3E and F), suggesting that Alisol A induces apoptosis in colorectal cancer cells. Meanwhile, treatment with Alisol A upregulated the accumulation of pyroptosis-associated factors, such as cleaved caspase 1, GSDMD and GSDME, in the HCT-116 and HT-29 cells (Fig. 3G and H), indicating that Alisol A stimulates pyroptosis in colorectal cancer cells.

Alisol A attenuates migration of colorectal cancer cells. The association of Alisol A with the migration of colorectal cancer cells was further assessed. The data showed that Alisol A suppressed the migration ability of the HCT-116 and HT-29 cells, in a dose-dependent manner (Fig. 4A and B). Consistently, the effect of Alisol A on epithelial-mesenchymal transition (EMT) markers, including E-cadherin, N-cadherin and Vimentin, were also analyzed. Western blot analysis demonstrated that Alisol A increased E-cadherin protein expression levels, but decreased N-cadherin and Vimentin protein expression levels in HCT-116 and HT-29 cells (Fig. 4C and D).
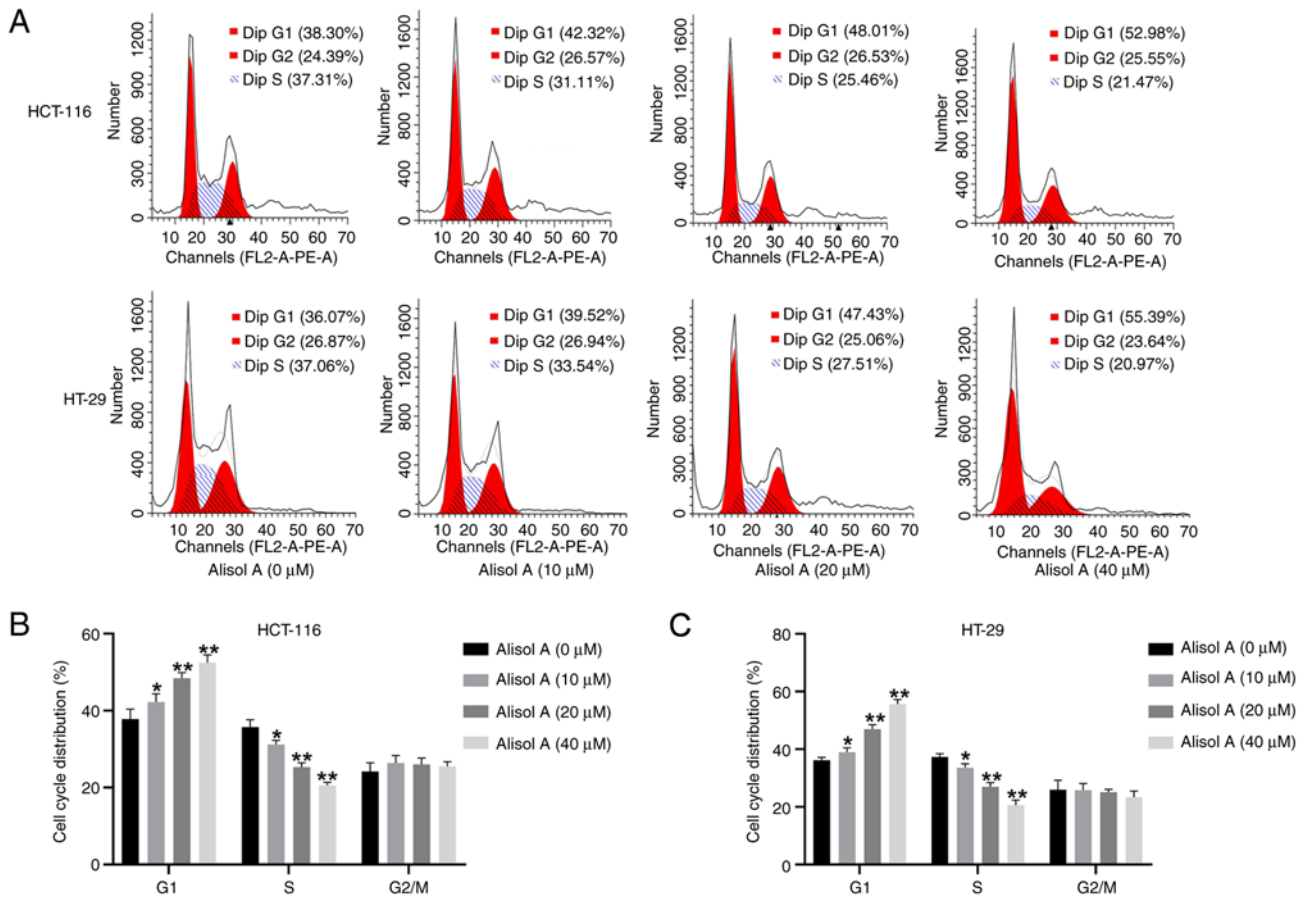
Figure 2. Alisol A induces cell cycle arrest of colorectal cancer cells. (A) Flow cytometry was performed in the HCT-116 and HT-29 cell lines to analyze the cell cycle following treatment with different concentrations of Alisol A, then the results were quantified in the (B) HCT-116 and (C) HT-29 cell lines. The results are presented as the mean ± SEM. n=3. *P<0.05, **P<0.01 vs. Alisol A (0 μM).

Alisol A increases the chemotherapeutic sensitivity of colorectal cancer cells to CIS. MTT results showed that CIS reduced HCT-116 and HT-29 cell viability, while co-treatment with CIS and Alisol A reinforced this effect (Fig. 5A and B). Moreover, the results from the colony formation assay also confirmed that either CIS or Alisol reduced HCT-116 and HT-29 colony number, while co-treatment with CIS and Alisol A could reinforce this effect (Fig. 5C). These results suggest that Alisol A increases the chemotherapeutic sensitivity of colorectal cancer cells to CIS.

PI3K/Akt signaling is involved in Alisol A-mediated colorectal cancer progression. Next, to investigate the potential mechanism underlying Alisol A-mediated colorectal cancer progression, bioinformatics analysis was performed using BATMAN and CTD. Venn diagram of intersected candidate pathways was plotted (Fig. 6A), and the seven pathways were then presented in Fig. 6B. Western blot analysis confirmed that Alisol A repressed the phosphorylation levels of PI3K, Akt, and mTOR in the HCT-116 and HT-29 cells (Fig. 6C and D). The Akt activator SC79 reversed the effect of Alisol A on the phosphorylation levels of Akt and mTOR (Fig. 7A). Moreover, treatment with Alisol A reduced viability and induced apoptosis in the HCT-116 and HT-29 cells, in which the Akt activator SC79 could reverse these phenotypes (Fig. 7B and C), indicating that PI3K/Akt signaling could

be associated with Alisol A-mediated colorectal cancer progression.

Discussion

Colorectal cancer is one of the most prevalent malignancies, leading to severe cancer-related mortality and morbidity worldwide (2). Although different strategies, such as chemotherapy and radiation therapy as well as surgery, have been used for the treatment of colorectal cancer, the overall survival rate of colorectal cancer patients is relatively low (16,17). Therefore, the development of innovative and practical treatment candidates is urgently required. Alisol A is a natural agent from Alismatis rhizome and has shown anti-cancer properties (15); however, the role of Alisol A in colorectal cancer remains unreported. In the present study, the effect and potential mechanisms of Alisol A on proliferation, cell cycle, apoptosis, pyroptosis, migration and invasion of colorectal cancer cells were assessed.

Previous studies have identified several functions of natural compounds in the treatment of colorectal cancer (18,19). It has been reported that resibufogenin represses metastasis and growth of colorectal cancer by targeting RIP3-related necroptosis (20). Epigallocatechin-3-gallate suppresses the stemness of colorectal cancer cells by inhibiting the Wnt/β-catenin signaling pathway (21). Pancratistatin attenuates colorectal
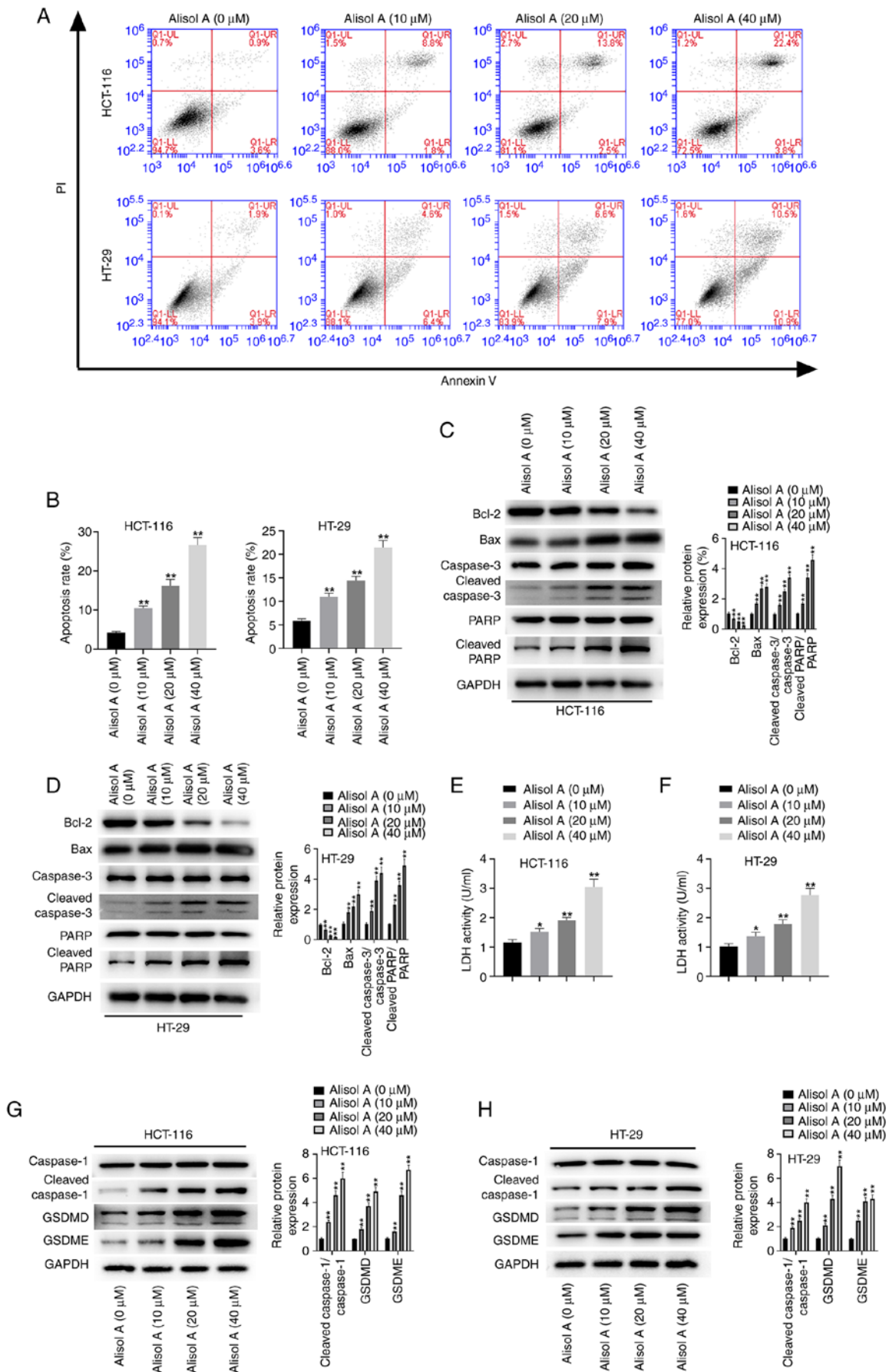
Figure 3. Alisol A induces apoptosis and pyroptosis of colorectal cancer cells. The HCT-116 and HT-29 cell lines were treated with different doses of Alisol A, then (A and B) flow cytometry, (C and D) western blot analysis, (E and F) ELISA and (G and H) western blot analysis were performed to analyze apoptosis, expression level of apoptosis-related proteins, LDH concentration and expression level of pyroptosis-related proteins, respectively. The results are presented as the mean ± SEM. n=3. *P<0.05, **P<0.01 vs. Alisol A (0 μM).
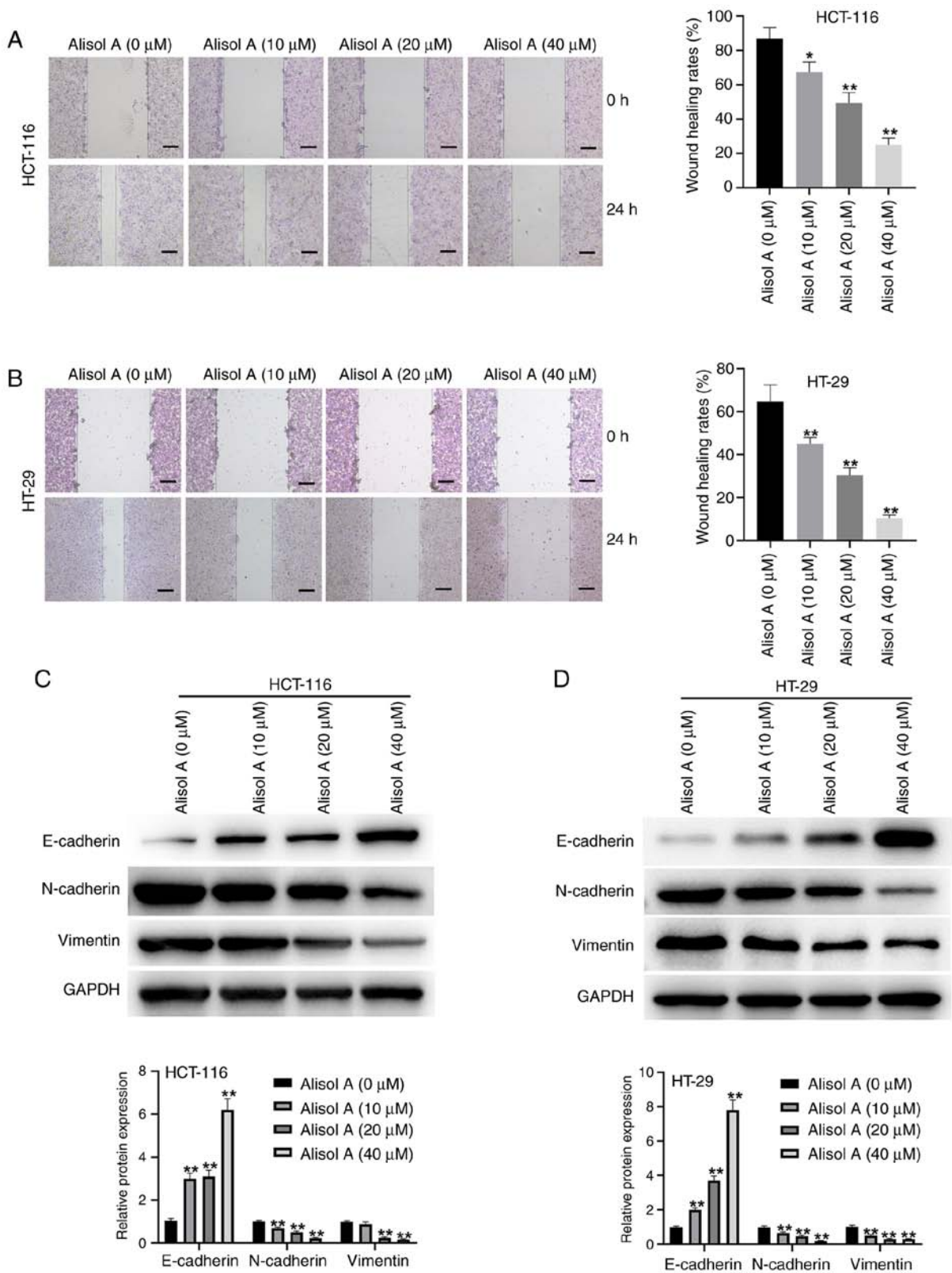
Figure 4. Alisol A attenuates migration of colorectal cancer cells. Wound healing assay was performed in the (A) HCT-166 and (B) HT-29 cell lines following treatment with different doses of Alisol A to measure migration. Western blot analysis was performed in the (C) HCT-116 and (D) HT-29 cell lines following treatment with different doses of Alisol A to measure the protein expression level of E- and N-cadherin and vimentin. The results are presented as the mean ± SEM. n=3, *P<0.05, **P<0.01 vs. Alisol A (0 μM).

cancer progression by initiating cell cycle arrest, autophagy and apoptosis (22). Xanthohumol decreases colorectal cancer cell proliferation by downregulating hexokinases II-regulated glycolysis (23). Deoxypodophyllotoxin inhibits colorectal cancer development by suppressing tumorigenesis and inducing apoptosis (24). Meanwhile, the constituents of Alismatis
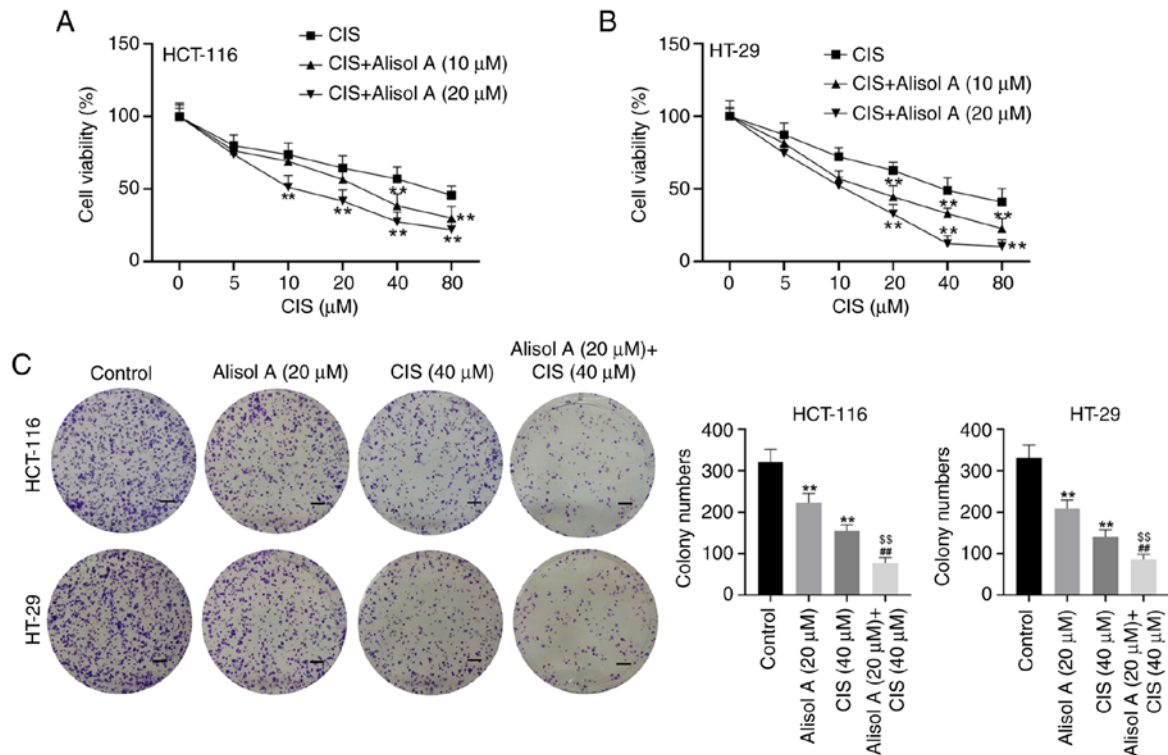
Figure 5. Alisol A increases the chemotherapeutic sensitivity of colorectal cancer cells to CIS. Cell viability was measured in (A) HCT-116 and (B) HT-29 cell lines treated with CIS alone or in combination with Alisol A (10 and 20 μM) using a MTT assay. **P<0.01 vs. CIS. (C) Colony formation assay was performed in the HCT-116 and HT029 cell lines treated with CIS or Alisol A alone or in combination to measure proliferation. The results are presented as the mean ± SEM. n=3. **P<0.01 vs. Control; ##P<0.01 vs. Alisol A; $$P<0.01 vs. CIS. CIS, cisplatin.
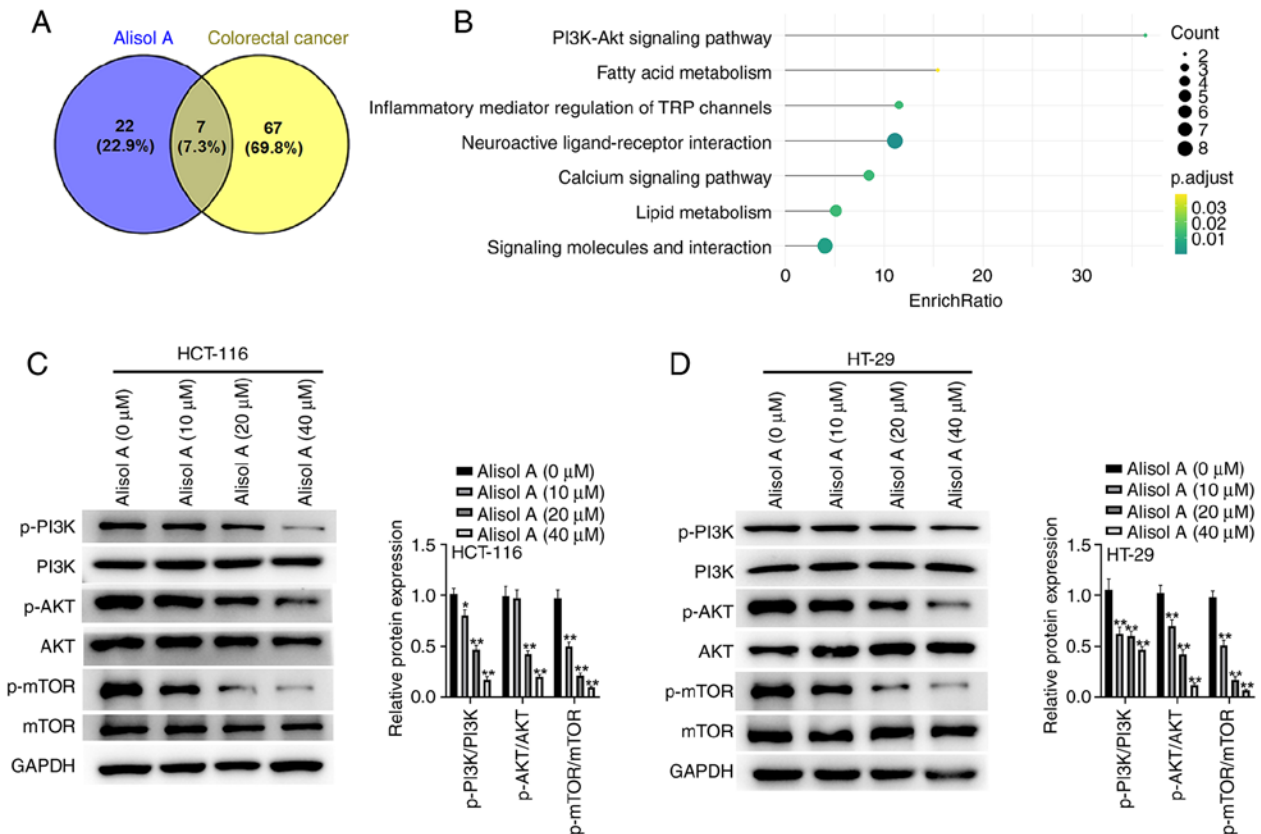

Figure 6. Alisol A inhibits PI3K/Akt signaling in colorectal cancer cells. Bioinformatics analysis using batman and CTD database on Alisol A and colorectal cancer. (A) Venn diagram of intersected candidate pathways was plotted and (B) seven pathways were then presented. (C) HCT-116 and (D) HT-29 cell lines were treated with different concentrations of Alisol A, then western blot analysis performed to determine the protein expression levels of p-PI3K, p-Akt and p-mTOR, PI3K, Akt and mTOR. Data are presented as the mean ± SEM. n=3, *P<0.05, **P<0.01 vs. Alisol A (0 μM). p, phosphorylated.
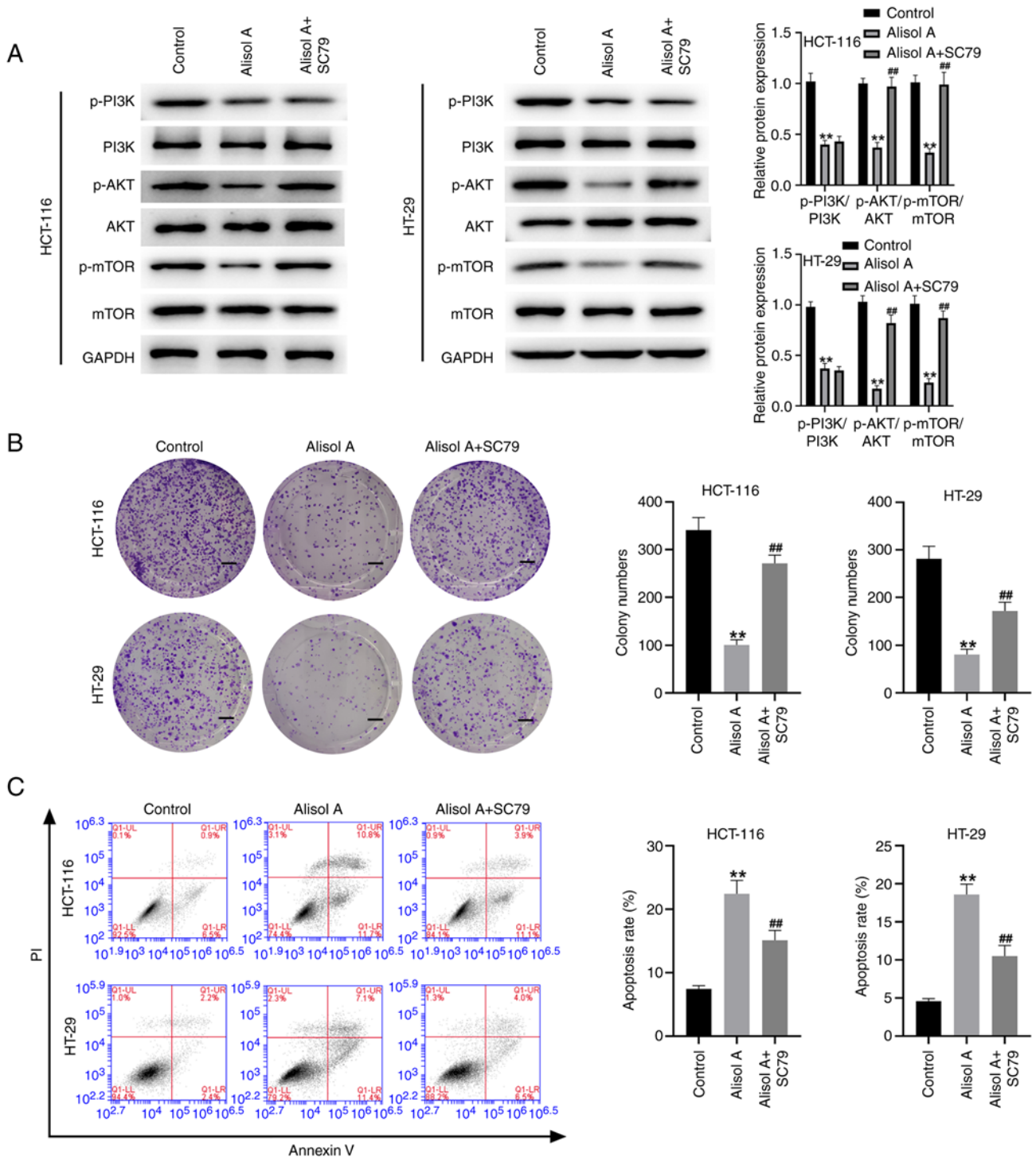
Figure 7. PI3K/Akt signaling is involved in Alisol A-mediated colorectal cancer progression. The HCT-116 and HT-29 cell lines were treated with Alisol A (40 μM) alone or in combination with SC79 (5 μM), then (A) western blot analysis, (B) colony formation assay (scale bar, 100 μm) and (C) flow cytometry was performed to analyse protein expression levels, proliferaiton and apoptosis, respectively. The results are presented as the mean ± SEM. n=3. **P<0.01 vs. control; ##P<0.01 vs. Alisol A.

rhizoma (zexie) have shown potential anti-cancer properties (12). The main protostane triterpenes of Alismatis Rhizom are mainly composed of Alisol A, alisol B, alisol B 23-acetate and Alisol A 24-acetate (25). In recent years, Alisol A is reported to play an important role in numerous diseases. For example, Alisol A represses metabolic disorders and high-fat diet-related obesity via AMPK/ACC/SREBP-1c signaling (26). Alisol A relieves arterial plaque in an apoE-knockout mouse

model by enhancing AMPK-SIRT1 signaling (14). Alisol A inhibits invasion, migration and proliferation of breast cancer cells (27). Moreover, Chen and Liu (28) have reported that Alisol A inhibits the proliferation, migration and invasion of nasopharyngeal carcinoma cells by inhibiting the Hippo signaling pathway. However, there has been no report regarding the effect of Alisol A on colorectal cancer. In the present study, Alisol A repressed proliferation, migration and induced cell

cycle arrest, apoptosis, and pyroptosis in colorectal cancer cells. Moreover, Alisol A increased the chemotherapeutic sensitivity of colorectal cancer cells to CIS. These data demonstrate an innovative anti-cancer effect of Alisol A in colorectal cancer progression, presenting valuable experimental basis for the function of Alisol A in the treatment of colorectal cancer. Specifically, pyroptosis has been reported to play critical roles in colorectal cancer development. Pyroptosis has been associated with reducing the effect of FL118 on the metastasis and growth of colorectal cancer cells (29). The present data showed that Alisol A stimulated pyroptosis of colorectal cancer cells. This indicates a new aspect of the anti-cancer function of Alisol A in colorectal cancer tumorigenesis.

PI3K/Akt signaling is essential for the development of colorectal cancer (30). Furthermore, it has been reported that IMPDH2 induces the progression of colorectal cancer by activating PI3K/AKT/FOXO1 and PI3K/AKT/mTOR pathways (31). DCLK1 enhances epithelial-mesenchymal transition in colorectal cancer via PI3K/Akt/NF-κB signaling (32). Curcumol represses the proliferation of colorectal cancer cells via miR-2/PTEN/PI3K/Akt signaling (33). GLI1 increases the stemness of colorectal cancer cells by PI3K/Akt/NF-κB signaling (34). FAT4 partially modulates autophagy and EMT by affecting PI3K/AKT signaling pathway in colorectal cancer cells (35). The present results indicated that SC79 abolished the effect of Alisol A on the phosphorylation levels of Akt and mTOR. In addition, SC79 could also reverse the functions of Alisol A on the viability and apoptosis in the colorectal cancer cells. This suggests that Alisol A exerts its anti-cancer effect on colorectal cancer by targeting PI3K/Akt signaling. However, the present study has some limitations, including a lack of data on the effect of Alisol A in other colorectal cancer cells and the effect of Alisol A *in vivo* was not evaluated. These limitations will be investigated further in future studies.

Alisol A induced an inhibitory effect on colorectal cancer progression by inactivating PI3K/Akt signaling. Alisol A may be used as a potential anticancer agent for the treatment of colorectal cancer.

Acknowledgements

Availability of data and materials

The dataset used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WH was involved in the conception and design of the study. WX and KW were involved in performing the experiments. BW and KB were involved in data analysis and interpretation. WH wrote the manuscript. WH and KB confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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