Progress in Microbes and Molecular Biology

HH PUBLISHER

Original Research Article

Mechanistic Insights into the Inhibition of Colorectal Cancer by BuShenFang through Adenomatous Polyposis Coli Expression and Wnt/β-catenin Pathway Regulation

Khang Wen Goh[1], Jie Ji[2*], Hiu Ching Phang[2], Xian Gu[3], Rajesh Sreedharan Nair[4], Xiaohui Wei[5], Mohammed Tahir Ansari[4], Phei Er Kee[6], Kai Bin Liew[2*]

Article History

Received: 25 October 2024;

Received in Revised Form: 10 December 2024;

Accepted: 18 December 2024;

Available Online: 15 January 2025

[1]Faculty of Data Science and Information Technology, INTI International University, Persiaran Perdana BBN Putra Nilai, 71800 Nilai, Negeri Sembilan, Malaysia, khangwen.goh@newinti.edu.my (KWG)

[2]Faculty of Pharmacy, University of Cyberjaya. Persiaran Bestari, 63000 Cyberjaya, Selangor, Malaysia, phanghiuching@gmail.com (HCP)

[3]Department of Oncology 5, Longhua Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 200032 Xuhui District, Shanghai, China, 13761347124@163.com (XG)

[4]School of Pharmacy, Faculty of Science and Engineering, University of Nottingham Malaysia, Semenyih, Selangor, Malaysia, Rajesh.Nair@nottingham.edu.my (RSN) and Tahir.Ansari@nottingham.edu.my (MTA)

[5]The MOE Key Laboratory for Standardization of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201210, China, xhweixh@163.com (XW)

[6]Biorefinery and Bioprocessing Engineering Laboratory, Department of Chemical Engineering and Materials Science, Yuan Ze University, Chungli, Taoyuan 320, Taiwan, pheier.kee@saturn.yzu.edu.tw (PEK)

*Corresponding authors: Kai Bin Liew and Jie Ji; Faculty of Pharmacy, University of Cyberjaya. Persiaran Bestari, 63000 Cyberjaya, Selangor, Malaysia; liewkaibin@cyberjaya.edu.my (KBL) and 2112-4733@st.cyberjaya.edu.my (JJ)

Abstract: Colorectal cancer (CRC) remains one of the leading causes of cancer-related mortality worldwide. Despite advances in chemotherapy and radiotherapy, significant side effects persist, prompting the exploration of alternative therapies such as traditional Chinese medicine (TCM). BuShenFang (BSF), a TCM formulation, is believed to exhibit anti-CRC effects, although its exact mechanism is unclear. This study aims to investigate the effects of BSF on CRC cells through the modulation of the Wnt/β-catenin pathway. The study utilized HCT116 and SW620 CRC cell lines, employing *in vitro* experiments including cell viability assays, colony formation, flow cytometry, and Western blot to examine the influence of BSF on cellular proliferation, apoptosis, migration, and the Wnt/β-catenin signaling pathway.

Results demonstrated that BSF significantly inhibited the proliferation of CRC cells in a dose-dependent manner. The apoptotic rate was markedly increased in the 20% BSF group, while colony formation, migration, and invasion capabilities of CRC cells were notably suppressed. Furthermore, Western blot results revealed that BSF enhanced adenomatous polyposis coli (APC) expression and inhibited β-catenin, c-Myc, and Cyclin D1, key proteins in the Wnt/β-catenin pathway. In conclusion, BSF exerts anti-CRC effects by modulating the Wnt/β-catenin pathway, suggesting its potential as a complementary therapeutic agent in CRC treatment. Future studies should focus on *in vivo* models to validate these findings.

Keywords: colorectal cancer ; Traditional Chinese medicine ; BuShenFang ; Wnt/β-catenin pathway

1. Introduction

In 2022, an estimated 20 million new cancer cases and 9.7 million cancer deaths occurred worldwide, of which more than 1.9 million new cases and over 900,000 deaths were attributed to CRC [1, 2]. Among various cancers, CRC ranks as the third most diagnosed cancer globally and the second leading cause of cancer-related death [3]. Specifically, the male morbidity rate and case fatality rate are ranked third and fourth, respectively, while the female morbidity rate and case fatality rate are ranked second and third, respectively [3, 4].

Although surgical treatment is the preferred approach, preoperative physicians must conduct a comprehensive analysis considering factors such as tumor location, stage, cell grade, patient tumor size, and bowel control ability to determine the appropriate surgical method [5]. Chemotherapy leverages the heightened sensitivity of tumor cells to chemical agents to selectively eradicate tumors. Various administration routes exist, including systemic intravenous administration and postoperative hyperthermic intraperitoneal chemotherapy. Similar to radiotherapy, chemotherapy can serve as an adjunct to surgical treatment for CRC [6]. Despite advancements in radiotherapy and chemotherapy regimens, patients continue to experience significant toxic side effects from long-term treatments. These adverse effects include gastrointestinal reactions such as retching, abdominal distension, and diarrhea; psychological issues like insomnia and depression; infections resulting from bone marrow suppression; and nerve damage, which can lead to limb numbness and abnormal sensations. Additionally, inflammatory reactions such as fever and phlebitis may occur, affecting the sensitivity of chemotherapy drugs to tumor cells [7].

In recent years, studies have demonstrated that traditional Chinese medicine (TCM) possesses specific characteristics and advantages in the treatment of CRC such as lesser side

effects, better recovery and improved quality of life, establishing TCM as a significant component of comprehensive CRC therapy. A prospective cohort study involving 312 patients with stage II and stage III CRC revealed that high exposure to TCM—defined as the application of syndrome differentiation to identify a specific pattern or syndrome that reflects the underlying imbalance in the body by TCM concept and treatment for more than one year—was associated with improved disease-free survival (DFS) (HR: 0.62, 95% CI: 0.39-0.98) and overall survival (OS) (HR: 0.31, 95% CI: 0.14-0.68) [8]. Banxia Xiexin Decoction (BXD), a formulation with a history of use in treating digestive system diseases in China for 1,800 years, contains high levels of liquiritigenin, rutin, baicalin, berberine, 6-gingerol, and ginsenoside Rh2. These components exhibit anti-inflammatory, immunomodulatory, anti-cancer, and antioxidant properties [9-12]. Both *in vitro* and *in vivo* experiments have confirmed that treatment of HCT116 cells with a mixture of BXD's primary components inhibits cell proliferation and migration. Furthermore, BXD significantly suppressed the growth of CRC subcutaneous xenograft tumors in nude mice, demonstrating its anti-CRC activity [13].

BuShenFang (BSF) is a kidney tonifying formulation, comprising *Pseudobulbus Cremastrae seu Pleiones*, *Fructus akebiae*, *Rehmannia glutinosa*, psoralen, and *Duchesnea indica*. *Rehmannia glutinosa* and psoralen are known for their functions in tonifying the kidneys and spleen, strengthening yang, and enhancing stomach function [14]. Meanwhile, *Pseudobulbus Cremastrae seu Pleiones*, *Fructus akebiae*, and *Duchesnea indica* contribute to clearing heat, detoxifying, eliminating carbuncles, and dispersing stagnation [15]. This TCM formulation has demonstrated potential to have anti-CRC effects. However, the actual mechanism of action is not determined yet. It is scientifically valuable to explore the mechanisms of action and anticancer signaling pathways, as this understanding could facilitate the establishment of clinical evidence for the use of BSF in treating colorectal cancer in the future.

The Wnt/β-catenin signaling pathway is a crucial driver in the occurrence and progression of CRC. Increased Wnt/β-catenin signaling is observed in nearly all CRC patients [16]. The adenomatous polyposis coli (APC) gene, a classic tumor suppressor and a key negative regulator of the Wnt/β-catenin signaling pathway, plays a vital role in the degradation complex of this pathway, thereby influencing the phosphorylation and degradation of β-catenin [17]. APC gene mutations are the most common mechanism for the activation of the Wnt pathway in CRC, with approximately 80% of colorectal cancers exhibiting somatic APC mutations [18]. Specifically, in the absence of activation of Wnt/β-catenin signaling, a complex comprising APC, GSK-3β, Axin, and other proteins facilitates the phosphorylation of β-catenin. However, when APC is mutated, the formation of this

complex is disrupted, thereby impairing the phosphorylation of β-catenin. This impairment prevents β-catenin from being catabolized and metabolized, resulting in an accumulation of β-catenin in the cytoplasm. As the concentration of β-catenin increases and reaches a critical threshold, it translocates into the cell nucleus. Once in the nucleus, free phosphorylated β-catenin can interact with T cell factor / lymphoid enhancer factor/ (TCF/LEF), thereby activating TCF transcriptional activity. This activation influences the transcription and translation processes of downstream target genes, leading to the production of corresponding proteins. Consequently, cells that are typically destined to differentiate and mature may instead retain stem cell characteristics, thereby expanding the stem cell niche. This aberration can contribute to the formation of adenomas and even malignant tumors [19]. Therefore, the Wnt/β-catenin signaling pathway is a focal point of current research.

This study aims to investigate the anti-colorectal cancer effect of BSF. BSF was utilized for *in vitro* experimental treatment to evaluate its biological effects on CRC cells and its influence on the Wnt/β-catenin signaling pathway. This research elucidates the positive role and therapeutic potential of BSF in CRC treatment at both theoretical and molecular levels, providing a framework for investigating and clarifying the mechanisms underlying the effects of traditional Chinese medicine compounds in the treatment of CRC.

2. Materials and methods

2.1. Preparation and Grouping of BSF

The BSF was processed by Bozhou Huikangtang Traditional Chinese Medicine Technology Co., Ltd. using a specific ratio: 15 g of *Pseudobulbus Cremastrae seu Pleiones*, 9 g of *Fructus akebiae*, 15 g of *Rehmannia glutinosa*, 12 g of Psoralen, and 15 g of *Duchesnea indica*. The above Chinese herbs were decocted with water to obtain 360ml of a solution with a concentration of 183 mg/ml.

The prepared stock solution of BSF was categorized into groups based on the following dosages: the control group (1 ml PBS containing 0 μl of BSF stock solution), the 2.5% BSF group (1 ml PBS containing 25 μl of BSF stock solution, representing 2.5% BSF), the 5% BSF group (1 ml PBS containing 50 μl of BSF stock solution, representing 5% BSF), the 10% BSF group (1 ml PBS containing 100 μl of BSF stock solution, representing 10% BSF), the 15% BSF group (1 ml PBS containing 150 μl of BSF stock solution, representing 15% BSF), and the 20% BSF group (1 ml PBS containing 200 μl of BSF stock solution, representing 20% BSF).

2.2. Cell cultures

Cell lines used were HCT116 and SW620 colorectal cancer cells, provided by Zhejiang Ruyao Biotechnology Co., Ltd. The cells were cultured in DMEM (Batch No.: 11995065, Gibco, USA) with 10% fetal bovine serum (Batch No.: 10099141, Gibco, USA) and 1% Penicillin-Streptomycin (Batch No.: 15140122, Gibco, USA) in humidity at 37°C, 5% CO2.

2.3. Detection of cell viability by CCK-8

Cell viability analysis was performed according to a previous report [20]. Briefly, CRC cells were plated into 96-well plate. 10 μl of CCK8 reagent (Batch No.: E606335-0500, BBI Life Sciences, Shanghai, China) was added to each well. The plate was incubated in a 37°C cell culture incubator in the dark for 40 min. Finally, a microplate reader (Batch No.: EPOCH2, Biotek, USA) was utilized to measure the absorbance value at 450 nm for data analysis.

2.4. Apoptosis was detected by flow cytometry

As previously reported by Shang et al. [21], the CRC cells were resuspended in 250 μl of 1× Binding Buffer and adjusted the concentration to 1×10^6 cells/ml. The 100 μl of the cell suspension was transferred into a 5 ml flow tube, then 5 μl of Annexin V-FITC and 5 μl of PI (Batch No.: E-CK-A217, Elabscience, Wuhai, China) were added, mixing gently. The mixture was protected from light and allowed to react at room temperature for 15 min. Finally, 400 μl of 1× Binding Buffer was added and mixed thoroughly, and the percentage of apoptosis was analyzed by flow cytometry (Model NO. Attune NXT, Thermo, USA) within 1 hour. The sum of Annexin V+/PI- (early apoptosis) and Annexin V+/PI+ (late apoptosis or necrosis) apoptotic cells was measured.

2.5. Transwell test was used to detect the migration and invasion ability of cells

As mentioned before by Pan et al. [22], 100 μL serum-free medium was added to the upper chamber, and 600 μl of culture medium containing 20% FBS (Batch No.: FBS-CE500, NEWZERUM, New Zealand) was placed into the lower chamber of the 24-well plate. The CRC cells were fixed with 4% paraformaldehyde (Batch No.: G1101-500ML, Servicebio, Wuhai, China) for 30 min. The cells were stained with 0.1% crystal violet (Batch No.: C0121-500ml, Beyotime Biotechnology, Shanghai, China for 30min. Randomly three fields of view were selected to observe the cells under a 100x microscope (Model NO. CKX41, OLYMPUS, Japan) and calculate the area of migrating or invading cells.

2.6. Colony formation test was used to detect the ability of a single cell to grow into a colony

The cells were plated into 6 cm culture dishes (Batch No.: 705001, NEST, Wuxi, China) at a density of 1,000 cells per dish. Once the cells reached a specific confluence, they were fixed using 4% paraformaldehyde. Following fixation, the cells were stained with 0.1% crystal violet for observation and imaging. The number of cells was then observed in each cell group [23].

2.7. Immunofluorescence detected the translocation of β-catenin into the nucleus

A total of 2.0×10^5 cells were seeded in a 24-well plate, followed by the addition of 4% formaldehyde to fix the samples for 30 min. Subsequently, a membrane rupture solution was added, and the samples were incubated for an additional 30 min to facilitate membrane rupture. The samples were then washed twice with PBS, after which a blocking solution was added and incubated for 30 min. Following the removal of the blocking solution, the samples were washed three times with TBST. Next, primary antibody (β-catenin, 1:200) was added and incubated overnight at 4 °C. After removing the primary antibodies, the samples were washed three times with TBST. A secondary antibody (Alexa Fluor 488-labeled goat anti-rabbit IgG, 1:500) was then added and incubated for 1 hour at room temperature in the dark. After removing the secondary antibody, the samples were washed three times with TBST. Finally, DAPI (1:10,000, Batch No.: C1002, Beyotime Biotechnology, Shanghai, China) was added and incubated for 5min, also in the dark, before washing twice with PBS and preparing the samples for microscopic examination [24].

2.8. Western Blot detection of downstream protein levels of Wnt/β-catenin signal in cells

RIPA lysis buffer (Batch No.: P0013B, Beyotime Biotechnology, Shanghai, China) was utilized to lyse the cells. The BCA Protein Assay Kit (Batch No.: P0006, Beyotime Biotechnology in Shanghai, China) was employed to measure and calculate the total protein concentration. Equal amounts of protein were separated using SDS-PAGE gels, and the gels were subsequently transferred to PVDF membranes (Batch No.: ISEQ15150, Millipore, USA). The PVDF membrane was soaked in a blocking solution of 5% skim milk in TBST for 40 min, followed by overnight incubation with the primary antibody at 4 °C. The primary antibodies used were APC (Item No.: 19782-1-AP), COX2 (Item No.: 66351-1-Ig), c-Myc (Item No.: 10828-1-AP), GSK3B (Item No.: 22104-1-AP), Cyclin D1 (Item No.: 60186-1-Ig), Axin1 (Item No.: 16541-1-AP), β-catenin (Item No.: 51067-2-AP) and p-GSK-3β (Item No.: 67558-1-lg) They were purchased from Proteintech Group, Inc. in Wuhai, China. The corresponding HRP-labeled secondary antibody (Alexa Fluor 488 labeled goat anti-rabbit IgG, Item No.: GB25303, Servicebio, Wuhai, China) was diluted with TBST at a ratio of

1:5000, then the PVDF membrane was immersed in the secondary antibody incubation solution and incubated on a shaker at 37°C for 1 h. Membranes were washed with TBST 3X, and analysis was performed using the ECL detection system [25].

2.9. Statistical analysis

All analyses were performed at least in triplicate. The experimental data were analyzed using SPSS version 27.0 statistical software, while GraphPad Prism version 9.0 was utilized for graphical representation. The experimental results are expressed as mean ± standard deviation (x ± SD). Comparisons between the two groups were conducted using an independent samples t-test. One-way analysis of variance (ANOVA) was employed for comparisons among multiple groups. A p-value of 0.05 was considered a statistically significant difference, among which * vs Ctrl $p < 0.05$, ** vs Ctrl $p < 0.01$, *** vs Ctrl $p < 0.001$, # vs xStAx-VHLL $p < 0.05$, ## vs xStAx-VHLL $p < 0.01$, ### vs xStAx-VHLL $p < 0.001$.

Expanded materials and instruments details are presented in the supplementary materials and raw blot images can be found in Figure S1 and S2.

3. Results

3.1. The concentration with the best anti-tumor effect of BuShenFang was selected

The groups were categorized based on BSF drug concentration and dosage, divided into the Ctrl group, 2.5% BSF group, 5% BSF group, 10% BSF group, 15% BSF group, and 20% BSF group. The results are illustrated in Figure 1. The cell viability of the Ctrl group, which received no drug treatment, remained unaffected. In contrast, BSF drug concentrations of 2.5%, 5%, 10%, 15%, and 20% exhibited significant inhibitory effects on human colorectal adenocarcinoma cells SW620 and HCT116 after 48 h. Notably, the inhibitory effect of BSF on SW620 and HCT116 colorectal cancer cells increased with dosage, demonstrating a dose-dependent relationship. At 20% BSF, SW620 and HCT116 cells exhibited the lowest relative cell viability compared to the other concentrations and dosages.

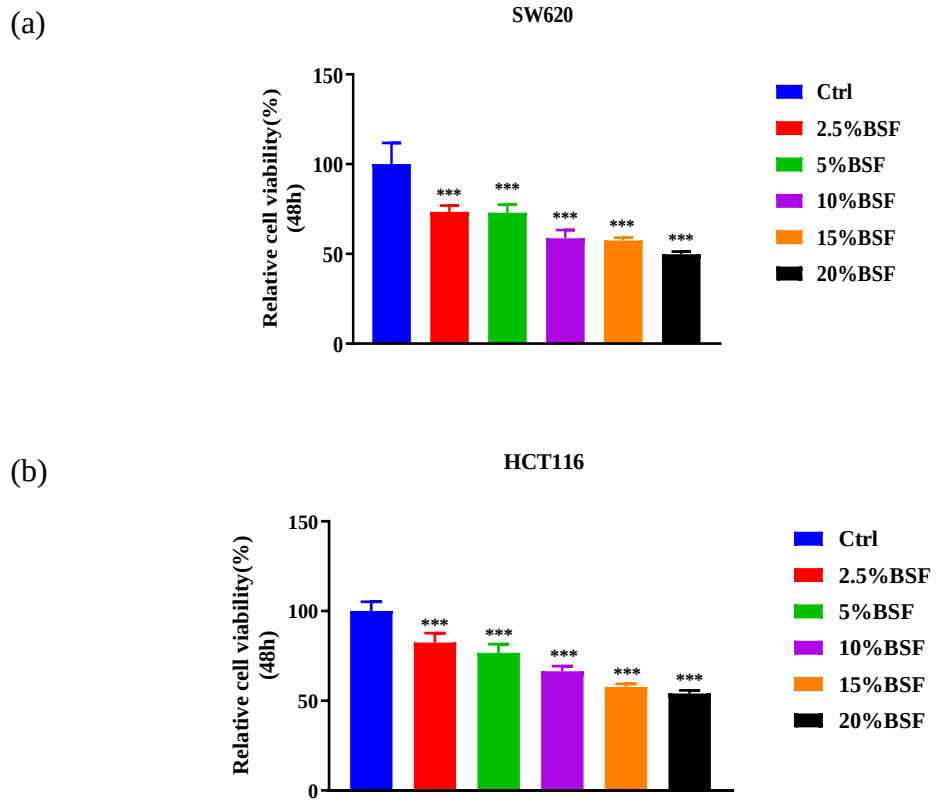

Figure 1. Effects of different concentrations of BuShenFang on (a) SW620 and (b) HCT116 cell viability. *** vs Ctrl, $p < 0.001$, as determined by cell activities detection using CCK-8 reagent, indicating statistically significance differences ($n = 5$ in each series).

3.2. BuShenFang inhibits SW620 cell viability

The dosing concentrations were established based on relative cell viability, the 20% BSF group was selected for further investigation. The xStAx-VHLL group was administered with 50 μM xStAx-VHLL as a positive control. xStAx-VHLL is a peptide-based PROTAC (Proteolysis Targeting Chimera) designed to selectively degrade β-catenin, a key protein in the Wnt signaling pathway. It consists of a β-catenin-targeting stapled peptide, xStAx, linked to a VHL-binding peptide, facilitating the recruitment of the von Hippel-Lindau (VHL) E3 ubiquitin ligase. This linkage promotes the ubiquitination and subsequent proteasomal degradation of β-catenin [26].

Over the course of the drug intervention, the viability of SW620 cells in both the 20% BSF and xStAx-VHLL groups decreased compared to the control group. Following 96h of treatment with 20% BSF and xStAx-VHLL, the relative cell viability of SW620 was significantly reduced, showing a statistically significant difference compared to the control group (Figure 2, $p < 0.01$, $p < 0.001$). There was no statistical significance difference between the 20% BSF group and the xStAx-VHLL group ($p>0.05$). These results indicate that 20% BSF significantly inhibits the growth of SW620 cells, demonstrating an inhibitory effect on cell growth.

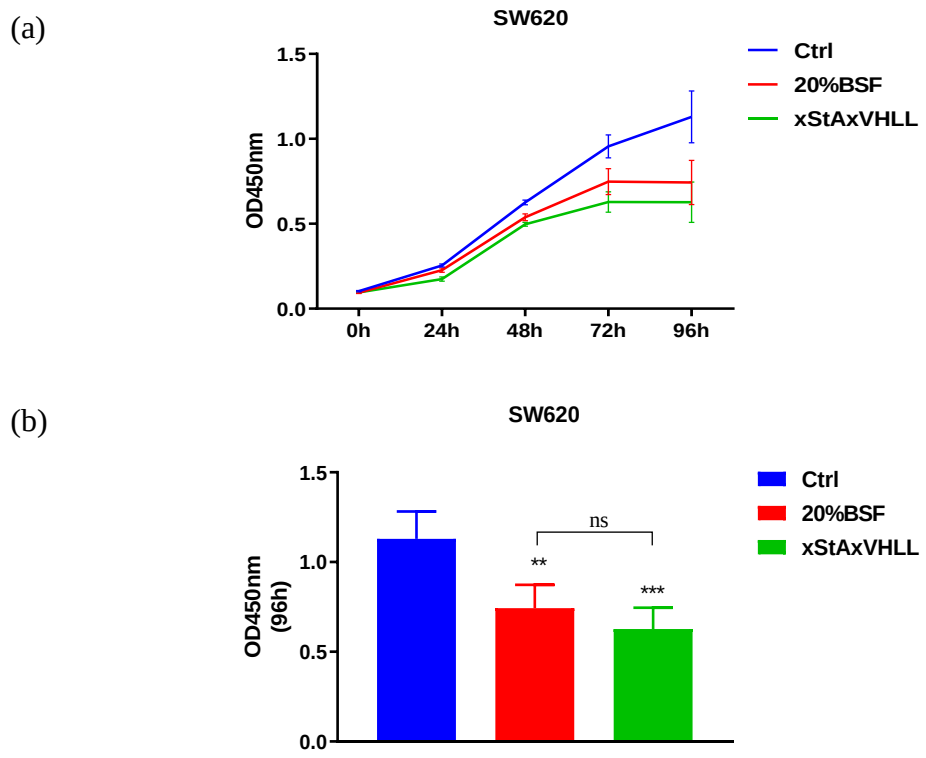

Figure 2. Effect of BuShenFang on cell viability of SW620. ** vs Ctrl, $p < 0.01$; *** vs Ctrl, $p < 0.001$, indicating statistically significance differences ($n = 5$ in each series).

3.3. BuShenFang inhibits HCT116 cell viability

As illustrated in Figure 3, the viability of HCT116 cells in both the 20% BSF group and the xStAx-VHLL group decreased over the duration of drug intervention compared to the Ctrl group. Following 96h of treatment with 20% BSF and xStAx-VHLL, the viability of HCT116 cells was significantly diminished, exhibiting a notable statistically significant difference relative to the Ctrl group ($p < 0.01$, $p < 0.001$). There was no statistical significance between the 20% BSF group and the xStAx-VHLL group ($p > 0.05$). These results indicate that 20% BSF can significantly inhibit the growth of HCT116 cells, thereby demonstrating an inhibitory effect on cell growth.

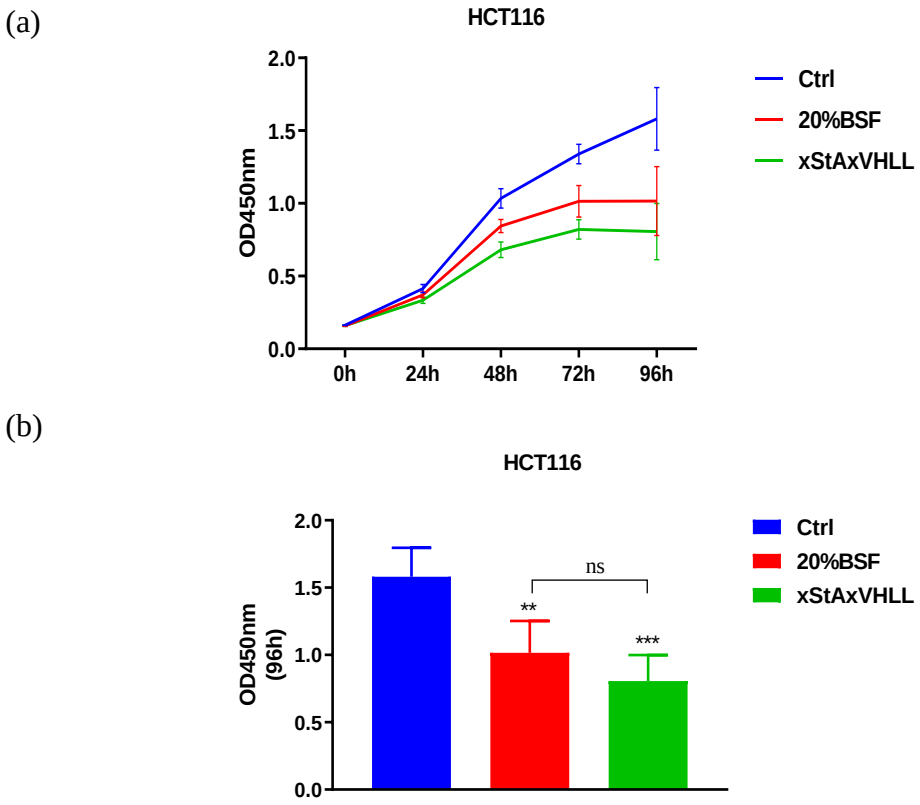

Figure 3. Effect of BuShenFang on cell viability of HCT116. ** vs Ctrl, $p < 0.01$; *** vs Ctrl, $p < 0.001$, indicating statistically significance differences ($n = 5$ in each series).

3.4. BuShenFang promotes apoptosis in SW620 cells

Flow cytometry was employed to assess the effects of apoptosis in SW620 cells treated with BSF and xStAx-VHLL at various concentrations. As illustrated in Figure 4, the results indicate that both 20% BSF and xStAx-VHLL induced cell apoptosis. Statistical analysis revealed a significant increase in the apoptosis rate of the 20% BSF group and the xStAx-VHLL group compared to the Ctrl group, with a statistically significant difference noted ($p < 0.001$). In comparison to the xStAx-VHLL group, the apoptosis rate in the 20% BSF group did not exhibit a significant change ($p > 0.05$). This indicates that 20% BSF can significantly enhance SW620 cell apoptosis.

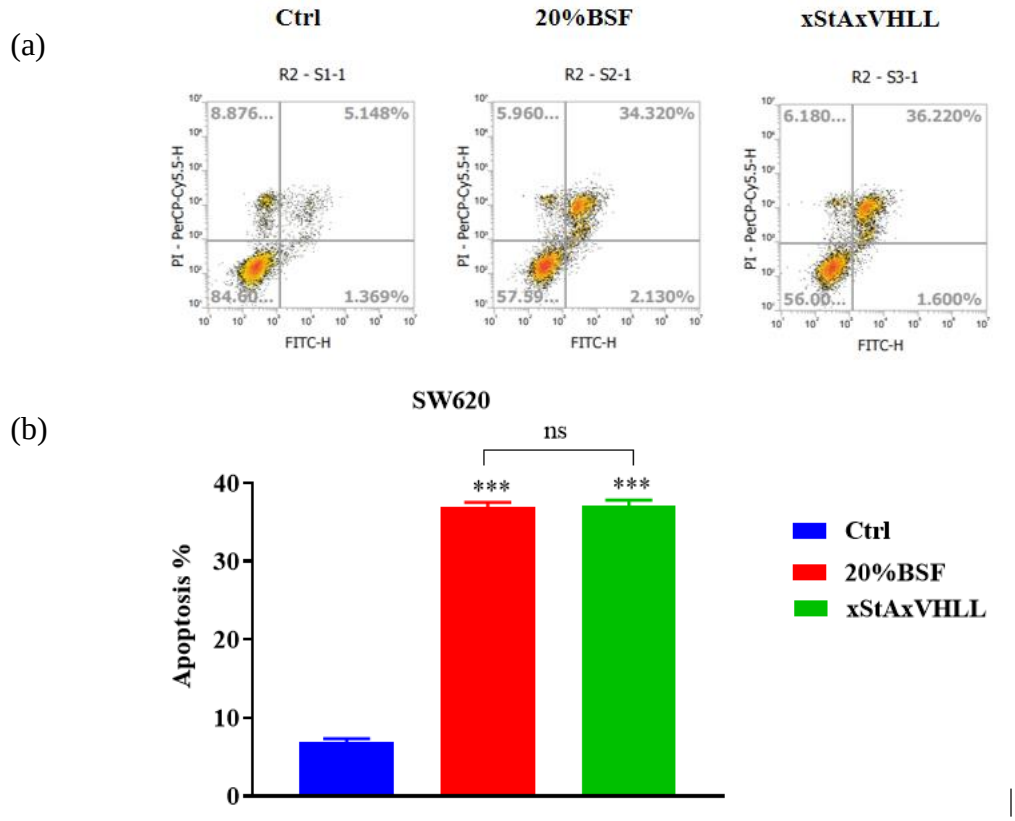

Figure 4. Effect of BuShenFang on apoptosis of SW620 cells. (a) Density plot of cells and (b) quantification of apoptosis%. *** vs Ctrl, $p < 0.001$, indicating statistically significance differences ($n = 3$ in each series).

3.5. BuShenFang promotes apoptosis in HCT116 cells

Flow cytometry was employed to assess the effects of apoptosis in HCT116 cells treated with varying concentrations of BSF and xStAx-VHLL. The results presented in Figure 5 indicate that both 20% BSF and xStAx-VHLL induced apoptosis in the cells. Statistical analysis revealed a significant increase in the apoptosis rate for both the 20% BSF group and the xStAx-VHLL group when compared to the Ctrl group, with the differences achieving statistical significance differences ($p < 0.001$). In comparison to the xStAx-VHLL group, the apoptosis rate of cells in the 20% BSF group did not exhibit a significant change ($p > 0.05$). This indicates that 20% BSF can significantly enhance HCT116 cell apoptosis.

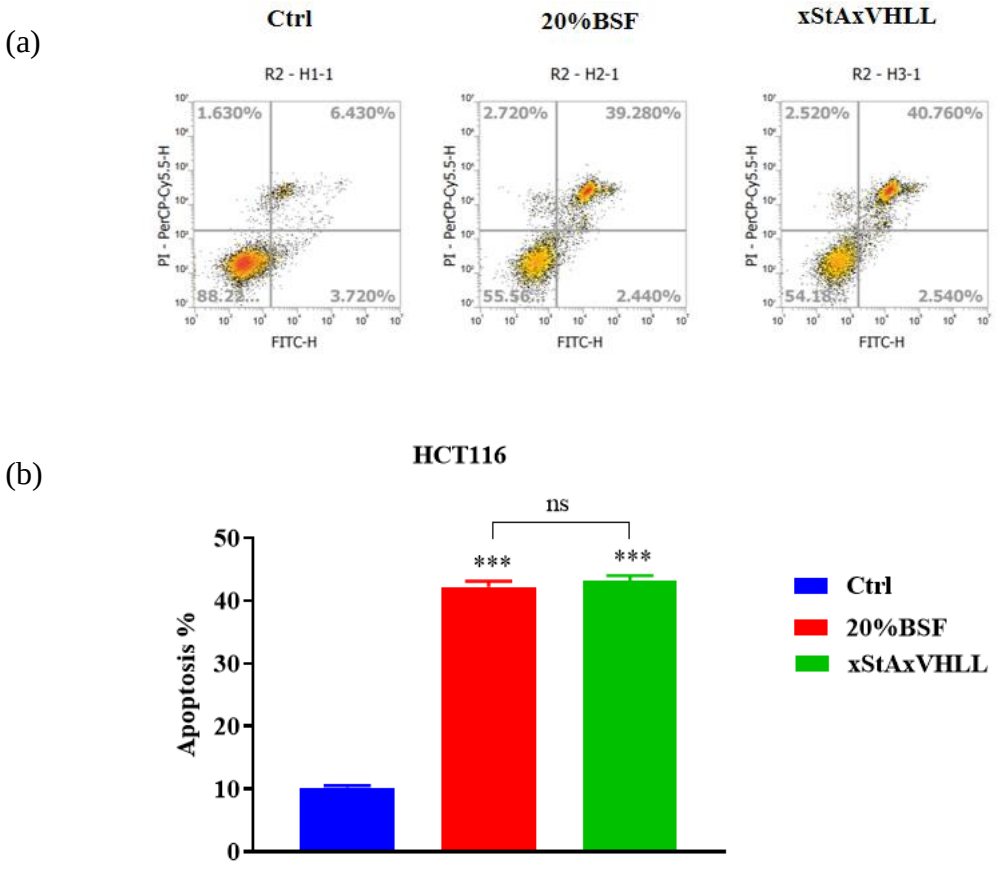

Figure 5. Effect of BuShenFang on HCT116 cell apoptosis. (a) Density plot of cells and (b) quantification of apoptosis%. *** vs Ctrl, p <0 .001, indicating statistically significance differences (n = 3 in each series).

3.6. BuShenFang inhibits colony formation in SW620 and HCT116 cells

The number of colonies formed serves as an indicator of cell proliferation activity. Various concentrations of BSF and xStAx-VHLL were applied to two human CRC cell lines, SW620 and HCT116. The experimental results (Figure 6) demonstrated that both the 20% BSF group and the xStAx-VHLL group significantly reduced colony formation in CRC cells. When compared to the control group, the differences were statistically significant differences ($p < 0.01$, $p < 0.001$). In comparison to the xStAx-VHLL group, the 20% BSF group exhibited no significant difference in the number of colony formation of SW620 cells ($p > 0.05$). This means that both xStAx-VHLL and BSF have comparable colony reduction ability. However, BSF showed a lower colony reduction effect in HCT116 cells compared to xStAx-VHLL and the difference is statistically significant differences ($p < 0.05$). These results indicate that 20% BSF significantly inhibits the clonogenic ability of both SW620 and HCT116 cells, with a more pronounced effect observed in SW620 cells.

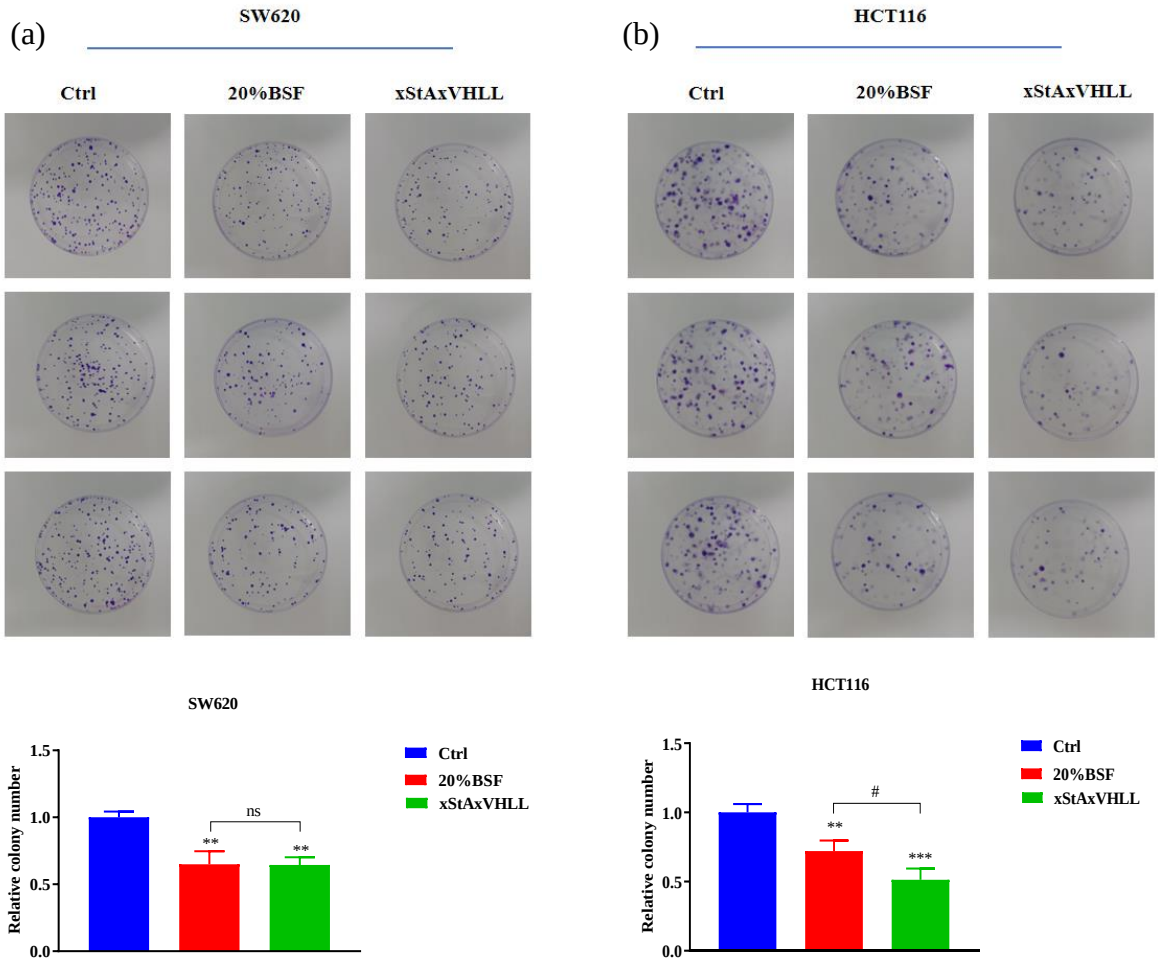

Figure 6. Effect of BuShenFang on the colony formation ability of (a) SW620 and (b) HCT116 cells. ** vs Ctrl, $p < 0.01$; *** vs Ctrl, $p < 0.001$; # vs xStAx-VHLL, $p < 0.05$, indicating statistically significance differences ($n = 3$ in each series).

3.7. BuShenFang inhibits the migration and invasion abilities of SW620 and HCT116 cells

The biological characteristics of tumors include invasiveness and metastasis, which are inherently linked to the capabilities of tumor migration and invasion. We employed the Transwell method to assess changes in cell migration and invasion abilities. The experimental results (Figure 7 and Figure 8) indicate that both the 20%BSF group and the xStAx-VHLL group significantly inhibited the migration and invasion of CRC cells. When compared to the Ctrl group, these differences were statistically significant differences ($p < 0.01$, $p < 0.05$). In comparison to the xStAx-VHLL group, the 20% BSF group exhibited no significant changes in the migration and invasion abilities of SW620 and HCT116 cells ($p > 0.05$). This indicates that 20% BSF can significantly inhibit the migration and invasion capabilities of SW620 and HCT116 cells.

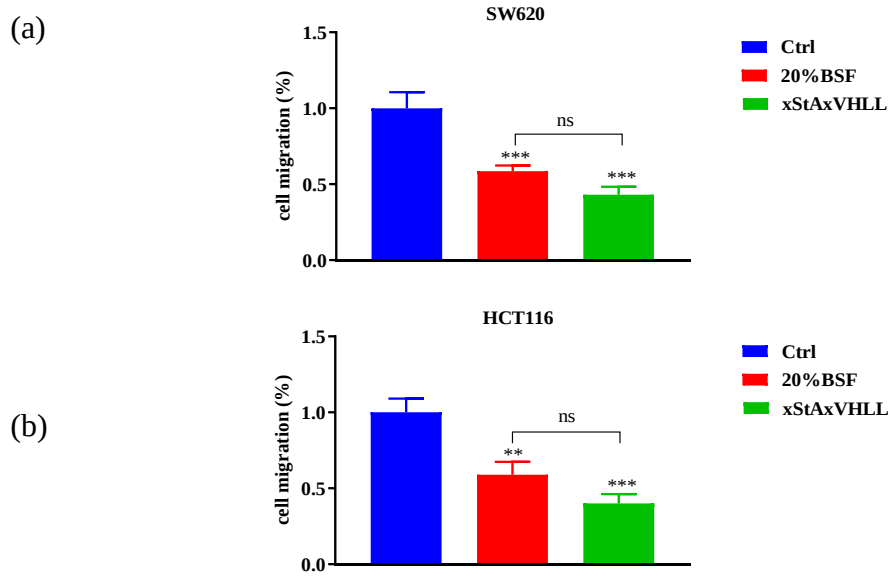

Figure 7. Effect of BuShenFang on the migration ability of (a) SW620 and (b) HCT116 cells. ** vs Ctrl, $p < 0.01$; *** vs Ctrl, $p < 0.001$, indicating statistically significance differences ($n = 3$ in each series).

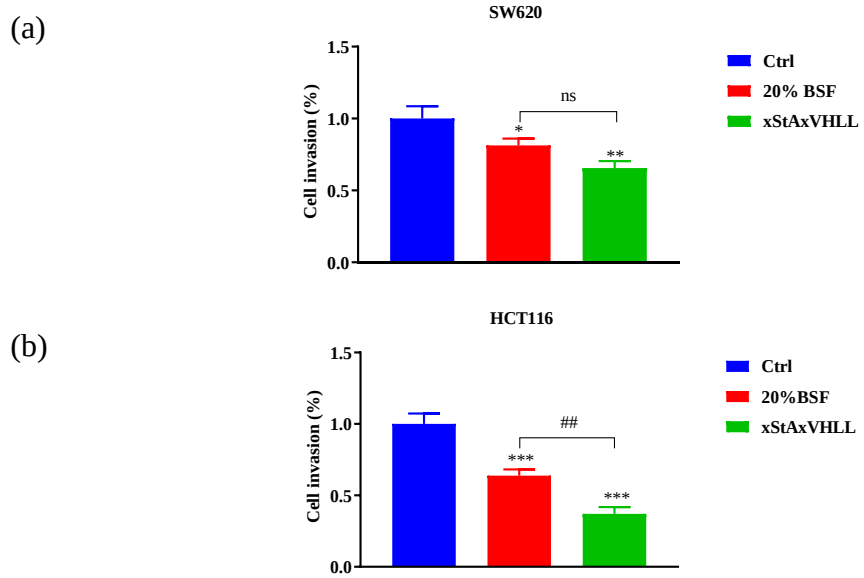

Figure 8. Effect of BuShenFang on the invasion ability of (a) SW620 and (b) HCT116 cells. * vs Ctrl, $p < 0.05$; ** vs Ctrl, $p < 0.01$; *** vs Ctrl, $p < 0.001$; ## vs xStAx-VHLL, $p < 0.001$, indicating statistically significance differences ($n = 3$ in each series).

3.8. Effect of BuShenFang on β-catenin in SW620 and HCT116 cells

Immunofluorescence was employed to assess the impact of varying concentrations of BSF and xStAx-VHLL on β-catenin levels in SW620 and HCT116 cells. The experimental results (Figure 9) indicated that β-catenin levels decreased following treatment in both the 20% BSF group and the xStAx-VHLL group, accompanied by a reduction in nuclear translocation.

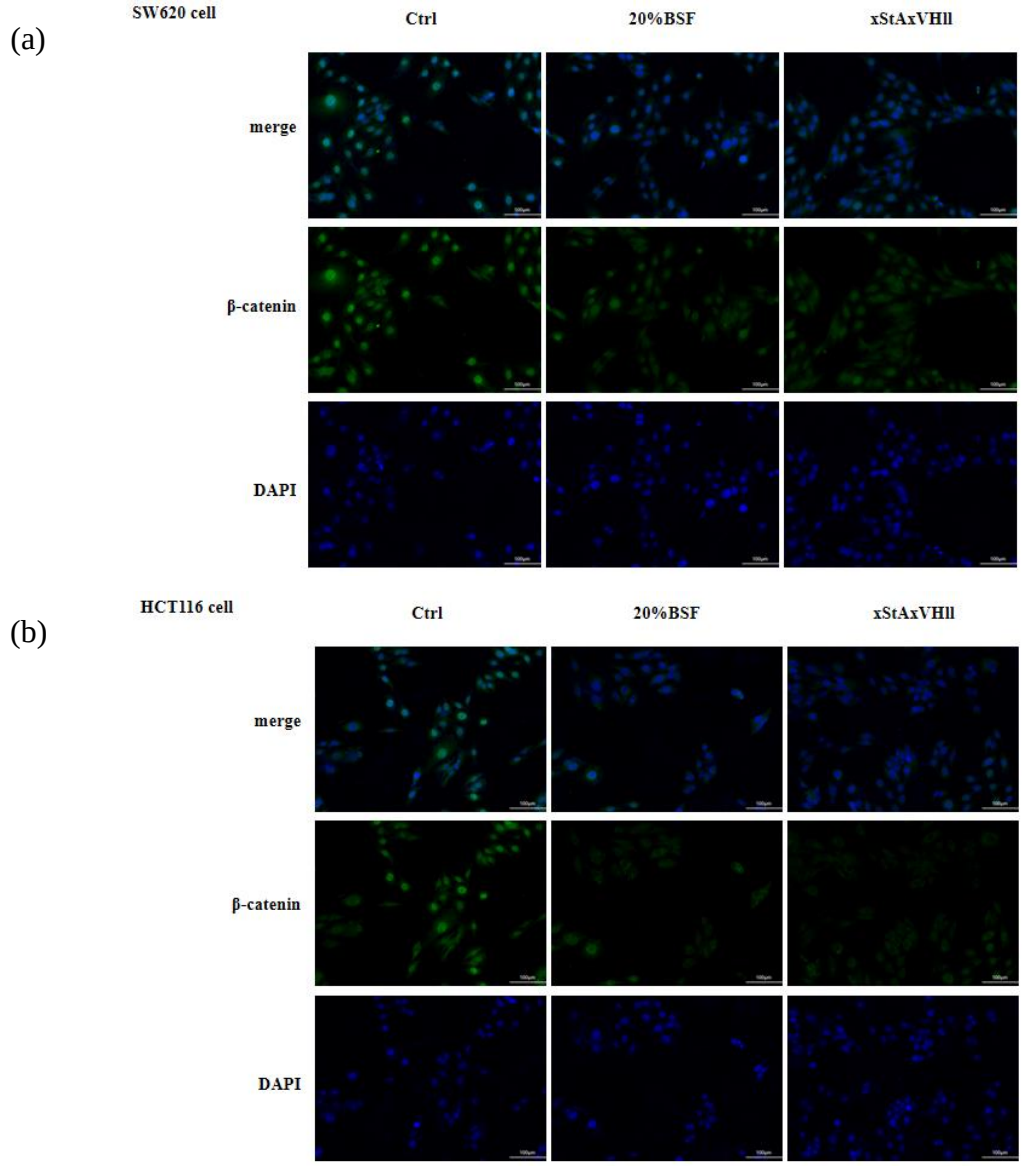

Figure 9. Immunofluorescence detection of the effect of BuShenFang on β-catenin molecule levels in (a) SW620 and (b) HCT116 cells (100X) (n = 3 in each series).

3.9. Effect of BuShenFang on the Expression of Wnt Signaling Pathway-Related Proteins in SW620 and HCT116 Cells

Western blotting was employed to investigate the effects of 20% BSF and xStAx-VHLL on Wnt-related proteins in SW620 and HCT116 cell lines. Figure 10 demonstrates that the 20% BSF group and the xStAx-VHLL group significantly inhibited the expression of β-catenin, p-GSK-3β, Axin1, c-myc, CyclinD1, and COX-2 proteins in both SW620 and HCT116 cells, while also increasing the expression of APC. Compared to the control group, these differences in protein expression were statistically significant ($p < 0.05$). In both SW620 cells, the expression levels of APC, β-catenin, p-GSK-3β and COX-2 in the 20% BSF group were statistically significant when compared to the xStAx-VHLL group ($p < 0.05$). In HCT116 cells, the expression of APC, β-catenin, p-GSK-3β, Axin1, c-myc, CyclinD1 and

COX-2 was statistically significantly different in the 20% BSF group compared to the xStAx-VHLL group ($p < 0.05$).

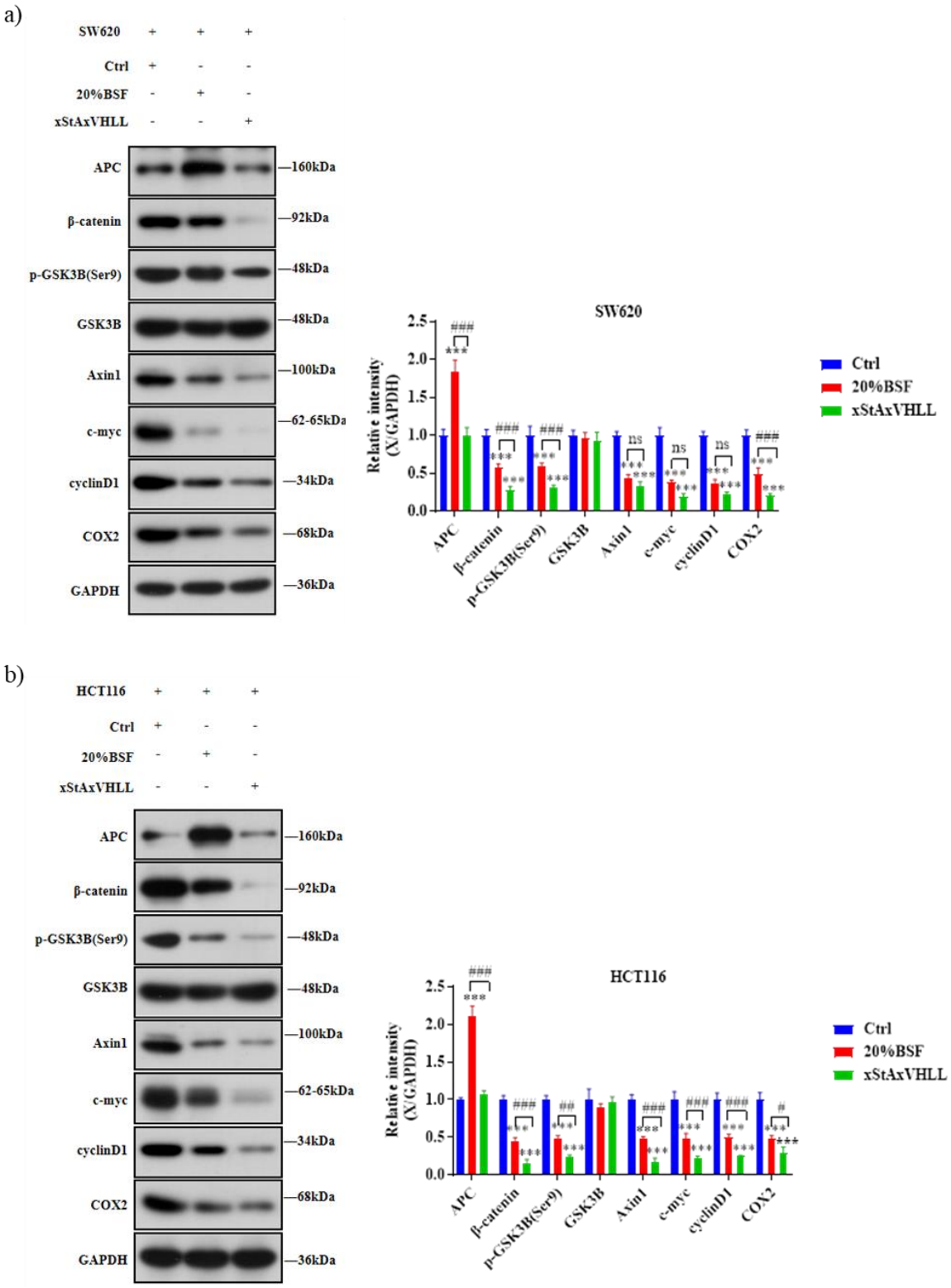

Figure 10. Effect of BuShenFang on the levels of related molecules in the Wnt signaling pathway in (a) SW620 and (b) HCT116 cells. *** vs Ctrl, $p < 0.001$; # vs xStAxVHLL, $p < 0.05$; ## vs xStAx-VHLL, pp < 0.01; ### vs xStAx-VHLL, $p < 0.001$, indicating statistically significance differences (n = 3 in each series).

4. Discussion

Traditional Chinese medicine posits that CRC is primarily caused by factors such as worry, anger, unhygienic food, chronic diarrhea, spleen failure, and the accumulation of warmth and heat in the intestines, leading to the formation of accumulations [27]. Treatment strategies largely focus on tonifying deficiencies and clearing the intestines. TCM practitioners typically diagnose CRC based on common symptoms and signs presented by patients through methods such as inspection, auscultation and olfaction, inquiry and palpation [28]. The predominant TCM syndrome types associated with CRC include spleen and kidney Yang deficiency, liver and kidney Yin deficiency, spleen deficiency with dampness, and phlegm and blood stasis[29]. These syndromes accompanied by spleen deficiency suggest a close relationship between CRC and spleen deficiency, often accompanied by kidney deficiency. These factors reflect the characteristics of traditional Chinese medicine syndromes associated with malignant tumors, which are characterized by a deficiency in the origin and an excess in manifestation, as well as a mixture of deficiency and excess [30]. Currently, the treatment of CRC typically involves surgical intervention followed by postoperative chemotherapy [31]. However, due to the multifaceted nature of CRC, which encompasses numerous genes, molecules, and pathways, the disease often develops drug resistance in its later stages, characterized by high rates of recurrence and metastasis. These challenges significantly complicate CRC treatment. For advanced CRC, the prevailing therapeutic approach combines chemotherapy with targeted therapy. Nevertheless, the emergence of drug resistance and severe side effects can vary based on the individual patient's disease characteristics and tolerance[32]. Consequently, the identification of multi-target, multi-pathway, and low-toxicity therapeutic agents is essential for advancing CRC treatment research.

The BSF utilized in this study comprises *Pseudobulbus Cremastrae seu Pleiones*, *Fructus akebiae*, *Rehmannia glutinosa*, psoralen, and *Duchesnea indica*. The *Pseudobulbus Cremastrae seu Pleiones* is characterized by its sweet, slightly pungent, and cool properties, and it is associated with the liver and spleen meridians. It is known for its ability to clear heat and detoxify, eliminate carbuncles, and disperse stagnation [33]. Recent pharmacological studies indicate that the extract of *Pseudobulbus Cremastrae seu Pleiones* exhibits anti-tumor effects through various mechanisms, including cytotoxicity, induction of tumor cell apoptosis, inhibition of tumor cell invasion and migration, modulation of immune function, and suppression of angiogenesis in tumor tissues [34]. Liu et al. [35] successfully isolated 11 distinct phenanthrene compounds from the ethanol extract of *Pseudobulbus Cremastrae seu Pleiones* tubers. *In vitro* experiments revealed that these compounds exert significant cytotoxic effects on colon cancer cells (HCT116), cervical cancer cells (HeLa), as well as

breast cancer cells (MCF-7 and MDA-MB-231), effectively inhibiting tumor cell proliferation. Additionally, Zhong et al. demonstrated that the extract of *Pseudobulbus Cremastrae seu Pleiones* may impede the migration and invasion of SW480 cells by downregulating the expression of AEG-1 and modulating the levels of E-cadherin, MMP-2, and MMP-9 proteins in human colorectal cancer SW480 cells [36].

Furthermore, *Fructus akebiae*, also known as August Zha, is characterized by its bitter and cold nature, influencing the liver, gallbladder, stomach, and bladder meridians. *Fructus akebiae* was used to regulate qi, disperse stagnation, and eliminate pathogenic factors, making it a common choice in the clinical treatment of tumors [37]. α-hedera saponin, a principal component of *Fructus akebiae* [38], has been recognized as a key quality control indicator for this drug in the 2020 edition of the Chinese Pharmacopoeia. Research indicates that α-Hedera saponin can induce lethal autophagy in human colon cancer cells (HCT116), ultimately leading to tumor cell apoptosis [39]. Additionally, Sun et al. [40] simulated the tumor inflammatory microenvironment *in vivo* and found that interleukin-6, at certain concentrations, significantly enhances the survival rate of colon cancer cells (SW620). Furthermore, α-Hedera saponin has been shown to inhibit the JAK2/STAT3 signaling pathway, reversing the epithelial-mesenchymal transition induced by interleukin-6, thereby inhibiting the progression and metastasis of colon cancer and demonstrating anti-tumor effects. Notably, α-Hedera saponin also plays a role in reversing tumor drug resistance and enhancing chemotherapy sensitivity. Chen et al. [41] demonstrated that α-hedera saponin can overcome hypoxia-mediated drug resistance in CRC by inhibiting the AKT/BCL2 pathway, both *in vivo* and *in vitro*. In human colon cancer models, α-hedera saponin effectively enhances the cytotoxicity of 5-FU, thereby improving its efficacy [42].

Rehmannia glutinosa is a significant medicinal herb known for nourishing yin and the kidneys. It has a sweet taste and is slightly warm in nature, associated with the liver and kidney meridians. Among the primary chemical components of *Rehmannia glutinosa*, polysaccharides, ionones, and phenylethanol glycosides have all demonstrated anti-tumor activity [43]. Polysaccharides not only induce an increase in the number of NK cells in peripheral blood and promote the activation of NK cells, but they also inhibit tumor growth [44]. Furthermore, they inhibit the proliferation and differentiation of tumor cells and induce apoptosis in these cells by enhancing T cell lethality, thereby exerting anti-tumor effect. Abd-Elbaset et al. [45] found that in a male Wistar rat model of hepatocellular carcinoma induced by diethylnitrosamine, ionone induced apoptosis in tumor cells by increasing the expression of Bax protein and reducing the expression of Bcl-2 protein, thereby exerting anti-tumor effects. Li et al. [46] reported that phenylethanoid glycosides can significantly inhibit tumor growth in mice and improve the survival rate of mice with a high tumor burden.

Psoralen is characterized as pungent, bitter, and warm in nature. Traditional Chinese medicine has long recognized that psoralen primarily functions to nourish kidney yang, thereby strengthening bones, relieving qi, alleviating asthma, warming the spleen, stopping diarrhea, and addressing issues related to insufficient kidney yang, deficiency and coldness in the lower yuan, as well as cold pain in the waist and knees [47]. Additionally, it is known to combat kidney insufficiency and deficiencies in qi, spleen, and kidney [47]. Recent studies have uncovered further biological effects of psoralen. Sun et al. [48] investigated the cytotoxic effect of psoralen on human colon cancer cells HT29 and HCT116, revealing that psoralen effectively induces Caspase-3/7-mediated apoptosis in these cells. Their findings suggest that psoralen-induced apoptosis may be mediated by reactive oxygen species and the c-Jun N-terminal kinase 1/2 (JNK1/2) pathway. Furthermore, the combined treatment of bakuchiol and TRAIL was shown to enhance the cleavage of Caspase-3/8/9 in HT29 and HCT116 cells. Additional investigation indicated that bakuchiol promotes the cleavage of caspase-3/8/9 in cancer cells through the activation of the reactive oxygen species/JNK signaling pathway, contributing to TRAIL-induced apoptosis [49]. Jin et al. [50] also reported that psoralen decreases the viability of human colon cancer SW480 cells while enhancing cell apoptosis by activating the NF-κB and Bcl-2/Bax protein signaling pathways. Moreover, psoralen demonstrates significant cytotoxic effects against human colon cancer HT29 cells [51]. Park et al. [52] investigated the effects of psoralen ethanol extract on human CRC cell lines HCT116 and SW480, finding that it downregulates cyclin D1 and cyclin-dependent kinase 4 (CDK4). Additionally, they examined the anti-cancer mechanisms in LoVo and HT29 cells, revealing that the psoralen-mediated degradation of cyclin D1 and CDK4 is dependent on extracellular regulated protein kinases 1/2 (ERK1/2) or glycogen synthase kinase-3β (GSK-3β).

Duchesnea indica, characterized as sweet, bitter, and cold in nature, is believed to target the lung, liver, and large intestine meridians. It is traditionally used to clear heat, cool blood, reduce swelling, and improve digestion. Modern pharmacological research indicates that *Duchesnea indica* possesses significant anti-tumor, antibacterial, immune-promoting, antihypertensive, and central nervous system inhibitory effects [50]. Research by Shoemaker et al. [53] demonstrated that *Duchesnea indica* exhibits the most pronounced inhibitory effect on cell proliferation at half inhibitory concentrations ranging from 208 to 419 μg/ml, indicating its strong anti-tumor properties.

Rehmannia glutinosa and psoralen are known for their ability to tonify the kidneys, strengthen yang, and enhance spleen and stomach functions [14]. In contrast, *Pseudobulbus Cremastrae seu Pleiones*, *Fructus akebiae*, and *Duchesnea indica* are effective in clearing heat, detoxifying, eliminating carbuncles, and dispersing stagnation [15], particularly in treating CRC associated with spleen and kidney deficiency syndrome.

This study initially employs varying concentrations of BSF (0, 2.5%, 5% , 10% , 15% , 20%) on CRC cell lines SW620 and HCT116. The results indicate that BSF significantly reduces the survival rate of CRC cells in a concentration-dependent manner ($p < 0.001$). Based on these experimental findings, subsequent experiments utilized a concentration of 20%. The infinite proliferation of tumor cells is a hallmark of malignant tumors. Results from CCK-8 assays and plate cell colony formation experiments demonstrated that the 20% BSF group and the xStAx-VHLL group significantly inhibited the activity and colony formation capacity of SW620 and HCT116 cells.

Apoptosis, a gene-controlled, cell-autonomous, and orderly form of cell death, regulates internal homeostasis, growth, and development by eliminating disordered cells [54]. Abnormal regulation of apoptosis is closely associated with tumor development and progression [55]. Flow cytometry analysis revealed that, compared to the control (Ctrl) group, both the 20% BSF group and the xStAx-VHLL group significantly enhanced apoptosis in CRC cells. The infiltration, invasion, and migration of tumor cells are hallmark characteristics of malignant tumors. Tumors that acquire these capabilities are particularly prone to local invasion and distant metastasis [56]. To assess the effects on cell migration and invasion, the Transwell method was employed. The results indicated that, relative to the Ctrl group, the 20% BSF group and the xStAx-VHLL group significantly reduced the migration and invasion capacities of CRC cells. The results from this study suggest that BSF exerts an inhibitory effect on both the proliferation and invasion of colon cancer cells.The Wnt/β-catenin pathway does not independently influence the occurrence and progression of a specific disease; rather, it collaborates with other signaling pathways such as TGF-β/BMP, PI3K/AKT, Notch, and Hedgehog [57-59]. These interactions are characterized by complex relationships. The Wnt pathway can directly or indirectly modulate the expression of downstream genes associated with these pathways, as well as the formation and secretion of related proteins, which is critically important for the onset and progression of the disease [60]. Therefore, the Wnt/β-catenin signaling pathway is the key point of current research. The Wnt/β-catenin signaling pathway consists of a multi-protein complex that includes Axin, GSK3-β, CK1α, APC, and other proteins. This complex is responsible for phosphorylating the β-catenin protein, which leads to its degradation. When the WNT ligand in the extracellular matrix binds to the Frizzled receptor and LRP5/6 on the cell membrane, it activates the Disheveled (DVL) protein [61]. This activation recruits a multi-protein complex composed of Axin, GSK3-β, CK1α, APC, and other proteins to interact with the receptor. The receptor binding inhibits the activity of GSK3-β, disrupting the function of the multi-protein complex and preventing the degradation of β-catenin protein [62]. Consequently, β-catenin accumulates in the cytoplasm and is transported to the nucleus, where it interacts with

the transcription factor TCF/LEF to activate the transcription of target genes within the Wnt/β-catenin pathway, such as cyclin D1, c-Myc, and COX-2 [19, 63]. The Wnt/β-catenin pathway plays a significant role in the occurrence and development of CRC by influencing cellular processes such as growth, proliferation, and differentiation. Mutations in components of this pathway can lead to its abnormal activation, resulting in the proliferation, invasion, and metastasis of tumor cells. Notably, CRC is a cancer type in which mutations in the Wnt/β-catenin pathway are particularly prevalent [64].

In the sequencing of 1,134 CRC samples, it was determined that Wnt activation was responsible for carcinogenesis in 96% of the samples [65]. The transcription factor β-catenin is a crucial component of the Wnt signaling pathway, and its abnormal regulation can precipitate early events in carcinogenesis [66]. Loss-of-function genetic screening has revealed that targeting the AKT/mTOR and Wnt/β-catenin pathways synergistically may be effective for treating acute myeloid leukemia (AML) with high PRL-3 phosphatase levels. The loss of β-catenin is linked to poor prognosis, diminished disease-free survival, reduced overall survival, and an increased likelihood of lymph node metastasis [67-69].

Clinical studies indicate that approximately 70% of sporadic and hereditary CRCs exhibit deletions or mutations in the APC gene. The degradation of APC can lead to the accumulation of β-catenin protein, subsequently activating the Wnt/β-catenin pathway and promoting cell proliferation, differentiation, and carcinogenesis [70]. In CRC, restoration of APC function can drive tumor cell differentiation and achieve sustained regression without recurrence [71]. Inhibiting APC loss and addressing many hallmarks of Wnt pathway dysregulation *in vivo* can impede tumor growth in the colon [72]. These findings underscore the critical role of APC mutations in the initiation and maintenance of intestinal tumors and highlight the Wnt pathway as a potential therapeutic target for CRC. Additionally, Axin and GSK3-β are implicated in CRC carcinogenesis, with mutations in the Axin1 or Axin2 genes identified in CRC and other cancers [73]. Several studies have indicated that both the expression and activity of GSK3-β are elevated in CRC [74]. GSK3-β contributes to increased cell proliferation levels and regulates chemoresistance through the NF-κB pathway [75]. Additionally, the target genes of the Wnt signaling pathway are implicated in the onset and progression of CRC. c-Myc, a proto-oncogene, has been shown to be overexpressed in CRC [76]. Cyclin D1 is a protein that plays a crucial role in cell proliferation; its overexpression can lead to uncontrolled cell proliferation, thereby facilitating cancer development [77].

Experimental treatments using 20% BSF and xStAx-VHLL on CRC cell lines SW620 and HCT116 revealed that, compared to the control group, both the 20% BSF group and the xStAx-VHLL group significantly enhanced the expression of APC protein while inhibiting

the expression of β-catenin, p-GSK-3β, Axin1, Cyclin D1, c-Myc, and COX-2 proteins. These findings suggest that BSF can inhibit the Wnt/β-catenin pathway to induce apoptosis in CRC cells and inhibit their proliferation, migration, and invasion.

5. Conclusion

In this study, two cell lines characterized by low APC expression, namely SW620 and HCT116, were utilized to screen for the concentration of traditional Chinese medicine, aiming to determine its effective dosage. Subsequently, the effects of BSF on SW620 and HCT-116 cells were evaluated from various perspectives, including cell viability, proliferation, apoptosis, migration, and invasion. In summary, BSF demonstrates the ability to inhibit the proliferation, migration, and invasion of CRC cells while inducing apoptosis, thereby exhibiting anti-tumor effects. Furthermore, BSF may significantly impede the occurrence and metastasis of CRC cells by activating APC and modulating the Wnt/β-catenin pathway. This segment of the research establishes a foundation for the validation of treatment in *in vivo* animal models and provides a scientific basis for reconciling the traditional Chinese medicine theory of the Bushen Recipe with contemporary medical understanding.

Author Contributions: Conceptualization: KWG and KBL. Formal analysis: KWG, JJ and HCP. Investigation: KWG, JJ and HCP. Writing – original draft preparation: JJ, HCP, XG and RSN. Writing – review and editing: XG, XW, MTA and PEK. Validation: XG, RSN, XW, MTA and PEK. Supervision: KBL. Funding acquisition: KBL.

Funding: This work was not supported by any funding agency.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bray F, Laversanne M, Sung H, *et al*. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2024; 74(3): 229-263.
2. Jardim SR, de Souza LMP, and de Souza HSP. The Rise of Gastrointestinal Cancers as a Global Phenomenon: Unhealthy Behavior or Progress? Int J Environ Res Public Health 2023; 20(4).
3. Sung H, Ferlay J, Siegel RL, *et al*. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. CA Cancer J Clin 2021; 71(3): 209-249.
4. Bray F, Ferlay J, Soerjomataram I, *et al*. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018; 68(6): 394-424.
5. Eto K, Kondo I, Kosuge M, *et al*. Enhanced Recovery After Surgery Programs for Laparoscopic Colorectal Resection May Not Need Thoracic Epidural Analgesia. Anticancer Res 2017; 37(3): 1359-1364.

6. Moriwaki T, Fukuoka S, Taniguchi H, *et al*. Propensity Score Analysis of Regorafenib Versus Trifluridine/Tipiracil in Patients with Metastatic Colorectal Cancer Refractory to Standard Chemotherapy (REGOTAS): A Japanese Society for Cancer of the Colon and Rectum Multicenter Observational Study. Oncologist 2018; 23(1): 7-15.
7. Li K, Xu G, Liu C, *et al*. Effect of a modified Banxia Xiexin decoction plus chemotherapy on stage III colon cancer. J Tradit Chin Med 2019; 39(2): 251-257.
8. Xu Y, Mao JJ, Sun L, *et al*. Association Between Use of Traditional Chinese Medicine Herbal Therapy and Survival Outcomes in Patients With Stage II and III Colorectal Cancer: A Multicenter Prospective Cohort Study. J Natl Cancer Inst Monogr 2017; 2017(52).
9. Li X, Chu S, Lin M, *et al*. Anticancer property of ginsenoside Rh2 from ginseng. Eur J Med Chem 2020; 203: 112627.
10. Li Y, Song K, Zhang H, *et al*. Anti-inflammatory and immunomodulatory effects of baicalin in cerebrovascular and neurological disorders. Brain Res Bull 2020; 164: 314-324.
11. Nouri Z, Fakhri S, Nouri K, *et al*. Targeting Multiple Signaling Pathways in Cancer: The Rutin Therapeutic Approach. Cancers (Basel) 2020; 12(8).
12. Samadi P, Sarvarian P, Gholipour E, *et al*. Berberine: A novel therapeutic strategy for cancer. IUBMB Life 2020; 72(10): 2065-2079.
13. Wang Y, Zhao T, Huang C, *et al*. Effect and mechanism of Banxia Xiexin decoction in colorectal cancer: A network pharmacology approach. Phytomedicine 2024; 123: 155174.
14. Zhang RX, Li MX, and Jia ZP. Rehmannia glutinosa: review of botany, chemistry and pharmacology. J Ethnopharmacol 2008; 117(2): 199-214.
15. Wang J, Wu M, Liu J, *et al*. An integrated strategy for quality control of Pseudobulbus Cremastrae seu Pleiones based on Q-marker. J Chromatogr A 2024; 1730: 465105.
16. Bian J, Dannappel M, Wan C, *et al*. Transcriptional Regulation of Wnt/β-Catenin Pathway in Colorectal Cancer. Cells 2020; 9(9).
17. Su LK, Vogelstein B, and Kinzler KW. Association of the APC tumor suppressor protein with catenins. Science 1993; 262(5140): 1734-7.
18. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012; 487(7407): 330-7.
19. Cheng X, Xu X, Chen D, *et al*. Therapeutic potential of targeting the Wnt/β-catenin signaling pathway in colorectal cancer. Biomed Pharmacother 2019; 110: 473-481.
20. Zeng K, Peng J, Xing Y, *et al*. A positive feedback circuit driven by m(6)A-modified circular RNA facilitates colorectal cancer liver metastasis. Mol Cancer 2023; 22(1): 202.
21. Shang A, Gu C, Wang W, *et al*. Exosomal circPACRGL promotes progression of colorectal cancer via the miR-142-3p/miR-506-3p- TGF-β1 axis. Molecular Cancer 2020; 19(1): 117.
22. Pan J, Liu F, Xiao X, *et al*. METTL3 promotes colorectal carcinoma progression by regulating the m6A–CRB3–Hippo axis. Journal of Experimental & Clinical Cancer Research 2022; 41(1): 19.
23. Liu F, Ou W, Tang W, *et al*. Increased AOC1 Expression Promotes Cancer Progression in Colorectal Cancer. Front Oncol 2021; 11: 657210.
24. Wang X, Rong S, Sun Y, *et al*. NDRG3 regulates imatinib resistance by promoting β-catenin accumulation in the nucleus in chronic myelogenous leukemia. Oncol Rep 2023; 50(2).

25. Chen Z, Li Y, He K, *et al*. CircGPRC5A enhances colorectal cancer progress by stabilizing PPP1CA and inducing YAP dephosphorylation. Journal of Experimental & Clinical Cancer Research 2023; 42(1): 334.
26. Liao H, Li X, Zhao L, *et al*. A PROTAC peptide induces durable β-catenin degradation and suppresses Wnt-dependent intestinal cancer. Cell Discovery 2020; 6(1): 35.
27. Zhang J, Wu Y, Tian Y, *et al*. Chinese herbal medicine for the treatment of intestinal cancer: preclinical studies and potential clinical applications. Molecular Cancer 2024; 23(1): 217.
28. Yan L, Jianzhong G, Huamiao Z, *et al*. Traditional Chinese Medicine symptom patterns in patients with colorectal carcinoma. Journal of Traditional Chinese Medicine 2018; 38(2): 299-308.
29. Wang Y-n, Zou M, Wang D, *et al*. An exploratory study on TCM syndrome differentiation in preoperative patients with colorectal cancer assisted by laboratory indicators. Heliyon 2022; 8(8).
30. Lin X, Yang X, Yang Y, *et al*. Research progress of traditional Chinese medicine as sensitizer in reversing chemoresistance of colorectal cancer. Front Oncol 2023; 13: 1132141.
31. Saif MW. Targeted agents for adjuvant therapy of colon cancer. Clin Colorectal Cancer 2006; 6(1): 46-51.
32. Sun J, Wei Y, Wang J, *et al*. Treatment of colorectal cancer by traditional Chinese medicine: prevention and treatment mechanisms. Front Pharmacol 2024; 15: 1377592.
33. Zhu J, Liu Z, Pu Y, *et al*. Green synthesized gold nanoparticles from Pseudobulbus Cremastrae seu Pleiones show efficacy against hepatic carcinoma potentially through immunoregulation. Drug Deliv 2022; 29(1): 1983-1993.
34. Fang Y, Ning A, Li S, *et al*. Polysaccharides Extracted from Rhizoma Pleionis Have Antitumor Properties In Vitro and in an H22 Mouse Hepatoma Ascites Model In Vivo. Int J Mol Sci 2018; 19(5).
35. Liu L, Li J, Zeng KW, *et al*. Five New Biphenanthrenes from Cremastra appendiculata. Molecules 2016; 21(8).
36. Zhong HC, Yang YQ, Li KZ, *et al*. The effects of extract of Shancigu on the migration and invasion of human colorectal cancer SW480 cell and its molecular mechanism. Anti-tumor Pharmacy 2023; 13(5): 590-596.
37. Zhou CJ, Zhou SQ, and Liu F. Medication Laws of LIU Zuyi in Treatment of Malignant Tumor Based on TCM Inheritance Support System. Chinese Journal of Information on Traditional Chinese Medicine 2019; 26(10).
38. Maciąg D, Dobrowolska E, Sharafan M, *et al*. Akebia quinata and Akebia trifoliata - a review of phytochemical composition, ethnopharmacological approaches and biological studies. Journal of Ethnopharmacology 2021; 280: 114486.
39. Sun J, Feng Y, Wang Y, *et al*. α-hederin induces autophagic cell death in colorectal cancer cells through reactive oxygen species dependent AMPK/mTOR signaling pathway activation. Int J Oncol 2019; 54(5): 1601-1612.
40. Sun D, Shen W, Zhang F, *et al*. α-Hederin inhibits interleukin 6-induced epithelial-to-mesenchymal transition associated with disruption of JAK2/STAT3 signaling in colon cancer cells. Biomed Pharmacother 2018; 101: 107-114.

41. Chen J, Xu J, Yang J, *et al*. α-hederin overcomes hypoxia-mediated drug resistance in colorectal cancer by inhibiting the AKT/Bcl2 pathway. Int J Oncol 2023; 62(3).
42. Bun SS, Elias R, Baghdikian B, *et al*. Alpha-hederin potentiates 5-FU antitumor activity in human colon adenocarcinoma cells. Phytother Res 2008; 22(10): 1299-302.
43. Jia J, Chen J, Wang G, *et al*. Progress of research into the pharmacological effect and clinical application of the traditional Chinese medicine Rehmanniae Radix. Biomedicine & Pharmacotherapy 2023; 168: 115809.
44. Xu L, Zhang W, Zeng L, *et al*. Rehmannia glutinosa polysaccharide induced an anti-cancer effect by activating natural killer cells. Int J Biol Macromol 2017; 105(Pt 1): 680-685.
45. Abd-Elbaset M, Mansour AM, Ahmed OM, *et al*. The potential chemotherapeutic effect of β-ionone and/or sorafenib against hepatocellular carcinoma via its antioxidant effect, PPAR-γ, FOXO-1, Ki-67, Bax, and Bcl-2 signaling pathways. Naunyn Schmiedebergs Arch Pharmacol 2020; 393(9): 1611-1624.
46. Li J, Li J, Aipire A, *et al*. Phenylethanoid Glycosides from Cistanche tubulosa Inhibits the Growth of B16-F10 Cells both in Vitro and in Vivo by Induction of Apoptosis via Mitochondria-dependent Pathway. J Cancer 2016; 7(13): 1877-1887.
47. Koul B, Taak P, Kumar A, *et al*. Genus Psoralea: A review of the traditional and modern uses, phytochemistry and pharmacology. J Ethnopharmacol 2019; 232: 201-226.
48. Sun C, Zhao L, Wang X, *et al*. Psoralidin, a natural compound from Psoralea corylifolia, induces oxidative damage mediated apoptosis in colon cancer cells. J Biochem Mol Toxicol 2022; 36(7): e23051.
49. Park MH, Kim JH, Chung YH, *et al*. Bakuchiol sensitizes cancer cells to TRAIL through ROS- and JNK-mediated upregulation of death receptors and downregulation of survival proteins. Biochem Biophys Res Commun 2016; 473(2): 586-92.
50. Jin Z, Yan W, Jin H, *et al*. Differential effect of psoralidin in enhancing apoptosis of colon cancer cells via nuclear factor-κB and B-cell lymphoma-2/B-cell lymphoma-2-associated X protein signaling pathways. Oncol Lett 2016; 11(1): 267-272.
51. Mar W, Je KH, and Seo EK. Cytotoxic constituents of Psoralea corylifolia. Arch Pharm Res 2001; 24(3): 211-3.
52. Park GH, Sung JH, Song HM, *et al*. Anti-cancer activity of Psoralea fructus through the downregulation of cyclin D1 and CDK4 in human colorectal cancer cells. BMC Complement Altern Med 2016; 16(1): 373.
53. Shoemaker M, Hamilton B, Dairkee SH, *et al*. In vitro anticancer activity of twelve Chinese medicinal herbs. Phytother Res 2005; 19(7): 649-51.
54. Obeng E. Apoptosis (programmed cell death) and its signals - A review. Braz J Biol 2021; 81(4): 1133-1143.
55. Molaei P, Savari M, Mahdavinezhad A, *et al*. Highlighting functions of apoptosis and circular RNAs in colorectal cancer. Pathology - Research and Practice 2023; 248: 154592.
56. Malki A, ElRuz RA, Gupta I, *et al*. Molecular Mechanisms of Colon Cancer Progression and Metastasis: Recent Insights and Advancements. Int J Mol Sci 2020; 22(1).
57. Guo X and Wang XF. Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res 2009; 19(1): 71-88.

58. Prossomariti A, Piazzi G, Alquati C, *et al*. Are Wnt/β-Catenin and PI3K/AKT/mTORC1 Distinct Pathways in Colorectal Cancer? Cell Mol Gastroenterol Hepatol 2020; 10(3): 491-506.
59. Krishnamurthy N and Kurzrock R. Targeting the Wnt/beta-catenin pathway in cancer: Update on effectors and inhibitors. Cancer Treat Rev 2018; 62: 50-60.
60. Zhao H, Ming T, Tang S, *et al*. Wnt signaling in colorectal cancer: pathogenic role and therapeutic target. Mol Cancer 2022; 21(1): 144.
61. He K and Gan WJ. Wnt/β-Catenin Signaling Pathway in the Development and Progression of Colorectal Cancer. Cancer Manag Res 2023; 15: 435-448.
62. Yu F, Yu C, Li F, *et al*. Wnt/β-catenin signaling in cancers and targeted therapies. Signal Transduct Target Ther 2021; 6(1): 307.
63. Chatterjee A, Paul S, Bisht B, *et al*. Advances in targeting the WNT/β-catenin signaling pathway in cancer. Drug Discov Today 2022; 27(1): 82-101.
64. Chen Y, Chen M, and Deng K. Blocking the Wnt/β-catenin signaling pathway to treat colorectal cancer: Strategies to improve current therapies (Review). Int J Oncol 2023; 62(2).
65. Yaeger R, Chatila WK, Lipsyc MD, *et al*. Clinical Sequencing Defines the Genomic Landscape of Metastatic Colorectal Cancer. Cancer Cell 2018; 33(1): 125-136.e3.
66. Zhang X, Wang L, and Qu Y. Targeting the β-catenin signaling for cancer therapy. Pharmacol Res 2020; 160: 104794.
67. Bruun J, Kolberg M, Nesland JM, *et al*. Prognostic Significance of β-Catenin, E-Cadherin, and SOX9 in Colorectal Cancer: Results from a Large Population-Representative Series. Front Oncol 2014; 4: 118.
68. Kamposioras K, Konstantara A, Kotoula V, *et al*. The prognostic significance of WNT pathway in surgically-treated colorectal cancer: β-catenin expression predicts for disease-free survival. Anticancer Res 2013; 33(10): 4573-84.
69. Veloudis G, Pappas A, Gourgiotis S, *et al*. Assessing the clinical utility of Wnt pathway markers in colorectal cancer. J buon 2017; 22(2): 431-436.
70. Kim S and Jeong S. Mutation Hotspots in the β-Catenin Gene: Lessons from the Human Cancer Genome Databases. Mol Cells 2019; 42(1): 8-16.
71. Dow LE, O'Rourke KP, Simon J, *et al*. Apc Restoration Promotes Cellular Differentiation and Reestablishes Crypt Homeostasis in Colorectal Cancer. Cell 2015; 161(7): 1539-1552.
72. Gay DM, Ridgway RA, Müller M, *et al*. Loss of BCL9/9l suppresses Wnt driven tumourigenesis in models that recapitulate human cancer. Nat Commun 2019; 10(1): 723.
73. Mazzoni SM and Fearon ER. AXIN1 and AXIN2 variants in gastrointestinal cancers. Cancer Lett 2014; 355(1): 1-8.
74. Domoto T, Pyko IV, Furuta T, *et al*. Glycogen synthase kinase-3β is a pivotal mediator of cancer invasion and resistance to therapy. Cancer Sci 2016; 107(10): 1363-1372.
75. Shakoori A, Ougolkov A, Yu ZW, *et al*. Deregulated GSK3beta activity in colorectal cancer: its association with tumor cell survival and proliferation. Biochem Biophys Res Commun 2005; 334(4): 1365-73.

76. Liu C, Rokavec M, Huang Z, *et al*. Salicylate induces AMPK and inhibits c-MYC to activate a NRF2/ARE/miR-34a/b/c cascade resulting in suppression of colorectal cancer metastasis. Cell Death Dis 2023; 14(10): 707.
77. Zhu X, Luo C, Lin K, *et al*. Overexpression of DJ-1 enhances colorectal cancer cell proliferation through the cyclin-D1/MDM2-p53 signaling pathway. Biosci Trends 2020; 14(2): 83-95.