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# The role of Solasonine and Solamargine on colon cancer cells: molecular and morphological analyses

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# Abstract

**Purpose of the study:** To observe the effects of solasonine and solamargine, alkaloids extracted from Solanum nigrum, on the proliferation of colon cancer cells and explore the possible related signaling pathways.

**Methods:** Three different colon adenocarcinoma cell lines HT-29, HCT116 and SW620, with increasing levels of invasiveness, were used. MTT was used to detect the effect of solasonine and solamargine on cell proliferation; Hoechst 33342 and pSIVA-IANBD / PI staining techniques were used to detect the presence of apoptosis; Western blotting was used to detect the expression of Caspase-3, Caspase-9, Bcl-2, IκB-α, p-IκB-α, p-NFκB P65 in the cytoplasm and NFκB P65 in the nucleus after treatment with solasonine and solamargine; finally, immunocytochemistry was used to observe the effects of the two molecules on NFκB P65 phosphorylation and nuclear translocation in HT-29 cells and HCT116 cells.

**Results:** Both solasonine and solamargine were able, *in vitro*, to: 1) inhibit the proliferation of HT-29, HCT116 and SW620 cells in a dose-dependent manner; 2) induce apoptosis of HT-29, HCT116 and SW620 cells, with a positive correlation with increasing drug concentration; 3) decrease the expression of p-IκB-α, p-NFκB, increase the expression of IκB-α and modify the expression of Caspase-3, Caspase-9 and Bcl-2; 4) reduce NFκB P65 phosphorylation in HT-29 and HCT116 cells and inhibit NFκB P65 nucleus translocation in the same cells.

**Conclusions:** Both tested molecules were capable, *in vitro*, to inhibit the proliferation and induce apoptosis of colon cancer cells in a dose-dependent manner and their effect is related to the NFκB pathway.



**Keywords:** solasonine; solamargine; colon cancer; traditional Chinese medicine

# Introduction

Cancer has always been a major killer threatening human health, and also a major problem perplexing the medical field. For hundreds of years, Western medicine has been constantly exploring the causes, mechanisms, clinical features, and detection indicators of cancer, and at the same time constantly searching for good medicine to overcome cancer. In China, because of its ancient civilization and long history, cancer was discovered and recorded as long as two thousand years ago. Since then, ancient Traditional Chinese Medicine (TCM) has considered, practiced, and documented the treatment of cancer. However, due to geographical and cultural differences, Western medicine and Chinese medicine did not combine in history.

With the development of modern oncotherapy, people have realized that the treatment of cancer requires a variety of means to cooperate. In China, TCM plays an indispensable role in the comprehensive treatment of cancer. As the auxiliary and supplement of major therapies such as surgery, chemotherapy and radiotherapy, both clinical observations and mechanism researches have confirmed the therapeutic effect of TCM on cancer. Meanwhile, it is the most accepted and easily realized way of integrating TCM and western medicine to study and utilize the effective components of Traditional Chinese Medications. Thus, to supplement the basic research basis, and to develop new effective drugs, this study took two components of an herb used in TCM to study their effect on colon cancer and the mechanism.

## 1 Colon Cancer

### 1.1 Cancer

Cancer is a disease caused by an abnormal mechanism of controlling cell growth and proliferation. It can destroy the structure and function of tissues and organs, cause necrosis and hemorrhage combined with infection, and the patient may eventually die due to organ failure. The tissue structure of a malignant tumor is heteromorphic: the tumor cells are more disordered, and lose the normal arrangement structure, hierarchy, or polar direction. The basic unit of carcinogenesis is cell: a variety of genetic and environmental oncogenic factors cause DNA damage, activate proto-oncogenes or inactivate tumor suppressor genes, and at the same time cause apoptosis and abnormal expression of DNA repair genes so that cells undergo a transformation. The transformed cells first show clonal proliferation, and after a long multi-stage evolution process, some of them expand unlimitedly. Through mutation, heterotypic cells with different characteristics are selectively formed, to obtain the ability of infiltration and metastasis, and finally, become

malignant tumor cells. The pathogenic factors of cancer can be divided into i) physical carcinogenic factors, such as X-rays and ionizing radiation; ii) chemical carcinogens, such as nitrites and aflatoxins; and iii) viral carcinogenic factors, such as hepatitis B virus, human papillomavirus.

In Italy, the number of incident cases of cancer in 2020 was 415,269, the estimated age-standardized incidence rate is 292.6 per 100,000, among them, the incidence of breast cancer, prostate cancer, colorectum cancer, lung cancer is at the forefront [1]. The number of cancer deaths in 2020 was 174,759, the estimated age-standardized mortality rate is 91.1 per 100,000, among them, the deaths rate of breast cancer, pancreas cancer, colorectum cancer, lung cancer is at the forefront [1]. In China, the number of incident cases of cancer in 2020 was 4,568,754, the estimated age-standardized incidence rate is 204.8 per 100,000, among them, the incidence of breast cancer, lung cancer, colorectum cancer, stomach cancer is at the forefront [1]. The number of cancer deaths in 2020 was 3,002,899, the estimated age-standardized mortality rate is 129.4 per 100,000, among them, the death rate of lung cancer, liver cancer, stomach cancer, oesophagus cancer is at the forefront [1]. Cancer has always been a major threat to human health and quality of life, but we are still on the way to find a cure for it.

### 1.2 Cancer therapies

Surgery, chemotherapy, radiotherapy, immunotherapy are the main adjuvant cancer therapies. Although the surgical techniques are constantly improving and innovating to reduce the resection range as far as possible, prolong the survival time and improve the quality of life of patients, adjuvant therapy is still essential and even increasingly important [2].

Chemotherapy as a kind of traditional oncotherapy is the use of chemical drugs to prevent the proliferation, invasion, metastasis of cancer cells, until the final killing of cancer cells. According to the mechanism of action, chemotherapeutic agents could be classified as: alkylating agents, such as nitrogen mustard, cyclophosphamide; antimetabolites, such as methotrexate, 5-fluorouracil (5-Fu); mitotic inhibitors, such as vincristine, paclitaxel; antibiotics, such as daunomycin, bleomycin; anthracyclines, such as doxorubicin, daunorubicin; topoisomerase II inhibitors, such as etoposide; nitrosoureas, such as carmustine, lomustine; antibodies, such as trastuzumab, bevacizumab; enzyme, such as asparaginase; DNA synthesis inhibitor, such as cisplatin, oxaliplatin; signal transduction inhibitor, such as imatinib, dasatinib; differentiation agents, such as all-trans retinoic acid, HDAC inhibitors; hormones and hormone antagonists, such as tamoxifen, abiraterone; proteasome inhibitors, such as bortezomib; DNA topoisomerase I inhibitors, irinotecan, topotecan; DNA repair inhibitor; arsenic trioxide; DNA methylation inhibitors, such as azacitidine; chimeric toxic protein [3].

Radiotherapy is the treatment of malignant tumors and some benign diseases by using one or more ionizing radiation. It can be used to shrink tumors before surgery, making them easier to remove or suppress the growth of remaining cancer cells after surgery. The most used directly ionized particles are electrons, and the most commonly used indirectly ionized particles are photons. Radiotherapy can be used for most cancers, and the most common is for brain tumors. The standard fractionation regimens are giving one fraction of 1.6–2.0 Gy per day, 5 days a week [4].

Cancer immunotherapy aims at activating the human immune system and killing cancer cells and tumor tissues using the autoimmune function. Different from surgery, chemotherapy and radiotherapy, immunotherapy targets not tumor cells and tissues, but the body's immune system. It can be divided into active immunotherapy, passive immunotherapy, and adoptive immunotherapy. Active immunotherapy uses antigenic vaccines, such as anti-mesothelin [5], to stimulate the body's immune system to produce anti-tumor immunity. Passive immunotherapy uses exogenous immune substances, such as PD-1/PD-L1 inhibitors [6], to induce an immune response, suppress signaling pathways, and deliver drugs to the lesion. Adoptive immunotherapy enhances the immune function of the recipient by transplants lymphocytes from the donor into the recipient, such as adoptive T cell therapy [7].

Despite the variety and innovation of cancer therapies, the survival and prognosis of patients are still unsatisfactory. Besides, the adverse reactions and side effects caused by treatment seriously affect the quality of life of patients even after a long time [8]. Thereupon, with the development of modern oncotherapy, people have realized that the treatment of cancer requires a variety of means to cooperate, for example, the rise of multi-disciplinary cancer care (a multi-disciplinary team should be composed of various specialists engaged in the diagnosis and treatment of cancer, including surgeons, physicians, oncologists, radiotherapists, radiologists, pathologists, psychologists, and sometimes surgeons of other specialties). In China, TCM plays an indispensable role in the comprehensive treatment of cancer. As the auxiliary and supplement of major therapies, both clinical observations and mechanism research have confirmed the therapeutic effect of TCM on cancer [9].

### 1.3 The molecular biology of cancer

The pathological process of cancer involves multiple stages, pathways and genes. The basic research on the molecular mechanism of carcinogenesis mainly focuses on the cloning and functional analysis of oncogenes, cell cycle regulation and signaling pathways. An oncogene is the intrinsic material basis of cell carcinogenesis. John Michael Bishop and Harold Eliot Varmus won the Nobel Prize in Physiology and Medicine in 1989 for their discovery of the oncogenes [10]. At

present, more than 100 oncogenes have been discovered, including growth factors (GFs), GF receptors, protein kinases, G proteins, transcription factors, and more than 20 tumor suppressor genes have been discovered, most of which are related to cell cycle or gene transcriptional regulation. The cell division process is driven and regulated by the cyclin-CDK-centered complex, and some tumor suppressor genes, such as the P53 gene [11], act as a checkpoint to participate in the regulation. The function of checkpoints is to monitor the state of each phase of the cell cycle. Common cancer signaling pathways include Just another kinase (JAK-STAT) [12], P53 [13], Nuclear factor κB (NFκB) [14], Ras-Raf [15], Mammalian target of rapamycin (mTOR) [16], Wnt [17], Bone morphogenetic protein (BMP) [18], and Mitogen-activated protein kinases (MAPKs) [19].

### 1.4 Colon cancer overview

There were 33,957 new cases of colon cancer in Italy in 2020 with a standardized incidence rate of 20.0 per 100,000, and 16,629 people died with an age-standardized mortality rate of 7.6 per 100,000 [1]. There were 306,078 new cases of colon cancer in China with a standardized incidence rate of 13.1 per 100,000, and 164,820 people died with an age-standardized mortality rate of 6.8 per 100,000 [1]. Dietary factors play an important role in the occurrence and development of colon cancer [20]. The vast majority of colon cancer is sporadic rather than familial, but familial adenomatous polyposis and hereditary nonpolyposis colon cancer are genetic syndromes prone to colon cancer [21]. Inflammatory bowel disease (IBD) is a risk factor for colon cancer, including ulcerative colitis and Crohn's disease [22].

Colon cancer diagnosis relies mainly on the clinical manifestations and auxiliary examination. Early colon cancer is asymptomatic or mild; the clinical manifestations of advanced colon cancer are related to tumor growth site and size; the main clinical symptoms of right colon cancer are anemia and abdominal mass; hematochezia, defecation habit change, and intestinal obstruction are the most common symptoms of left colon cancer. Barium double colonic radiography and barium enema can show the pathological changes and intestinal peristalsis in the intestinal cavity, which is of great value for the diagnosis of colon cancer. Ultrasonography, CT, and MRI can not only assist in the diagnosis of colon cancer but also show tumor invasion, lymph node and distant organ metastasis. The tumor markers such as carcinoembryonic antigen (CEA) and CA19-9 also have a suggestive effect on the diagnosis of colon cancer, but the "gold standard" for the diagnosis of colon cancer is still pathological biopsy.

1.5 Therapies for colon cancer

Surgery is currently the most effective treatment for resectable nonmetastatic colon cancer, mainly including endoscopic, laparoscopic, and open surgery. These approaches tend to preserve the morphology and function of the affected tissues and organs while improving the survival rate. The standard way is to remove the primary tumor and its associated mesentery.

Colon cancer patients may receive adjuvant chemotherapy based on the presence of high-risk factors for tumor recurrence. The internationally recognized standard chemotherapy regimens mainly include FOLFOX (oxaliplatin + 5-Fu + calcium linoleate) and XELOX (capecitabine + oxaliplatin). Irinotecan was previously thought to be effective for metastatic colon cancer, but recent studies [23, 24] have suggested that, in the FOLFIRI regimen (Irinotecan + 5-Fu + calcium linovolate), it does not affect disease-free survival (DFS) and overall survival (OS) in patients and also increases the toxicity of the regimen, so Irinotecan should not be used in postoperative adjuvant chemotherapy for colon cancer. Molecularly targeted therapy drugs used in colon cancer treatment are usually bevacizumab [25] (anti-vascular endothelial growth factor) and cetuximab [26] (anti-human and mouse chimeric epidermal growth factor receptor). These two drugs can be used in combination with cytotoxic agents for the treatment of metastatic colon cancer.

Postoperative radiotherapy has also been used as adjuvant therapy for colon cancer in combination with 5-Fu chemotherapy. However, clinical studies have shown that postoperative adjuvant chemotherapy plus radiotherapy does not significantly improve the prognosis, so the combination of chemotherapy and radiotherapy is no longer used [27].

Adjuvant therapies for colon cancer have improved patient survival, but these regimens are highly toxic and can cause myelosuppression, diarrhea, and dose-dependent neurotoxicity [28]. In a clinical observation of 42 patients receiving postoperative oxaliplatin plus 5-Fu chemotherapy for colon cancer, 11 patients experienced grade 3 hematologic toxicity [29]. Patients (aged <70 years) treated with surgery or surgery + chemotherapy both reported significantly more insomnia, diarrhea and financial problems (finance is also an important index of the quality of life of cancer patients, and the cost of overcoming side effects often causes financial stress) compared with an age and sex-matched normative population [30]. At the same time, considering that multiple drug resistance (MDR) is often the main cause of chemotherapy failure, these drug options are very limited when patients develop drug resistance. In these cases, it is necessary to identify new appropriate therapy or developing new drugs, and in this respect, TCM may be able to help.

## 2 TCM treatment of colon cancer

### 2.1 Traditional Chinese Medicine

TCM was born in a primitive society. The theory of TCM was formed around 500 BC with the emergence of anatomy and medical branches and has been summarized and developed in successive dynasties [31]. It has a profound impact on the Chinese-character cultural circle countries. For example, Japanese Kampo medicine, Korean medicine, Vietnamese medicine are all developed based on TCM. Under the guidance of simple materialism and spontaneous dialectics in ancient times, TCM is a medical theory system gradually formed and developed through long-term medical practice.

Unlike modern medicine, TCM theory tends to interpret physiology and pathology macroscopically. TCM views the human body as a whole, an interconnected system made up not just of physical structures such as organs, but also of emotions, spirit, minds which each play different roles, forming a complete human individual [32]. By adjustment and change of the whole, huge populations have been managed medically for thousands of years. TCM believes that human beings derive from and adapt to the natural environment as part of the natural ecosystem. The human body forms a smooth circulation system, similar to the ecological system, including the circulation of “qi” (an abstract concept, the most basic substance that constitutes the human body and maintains its vital activities, it has the functions of pushing, warming, defense, fixation, gasification and nutrition), blood and “essence” (the internal humors and normal secretions of the organs, the generic name of all normal fluids of the body) as shown in Figure 1. The theoretical system of TCM is vast and complete, with multiple perspectives. Some aspects of the current immune, hormonal, psychological disease and intestinal flora theories in modern mainstream medicine are gradually coinciding with the theory of TCM, such as the introduction of “microenvironment” [33], “rehabilitation”, and “phylaxiology” concepts. Modern medicine, which is mainstream globally, and TCM, which has supported the Chinese health system for thousands of years, can be effectively combined.

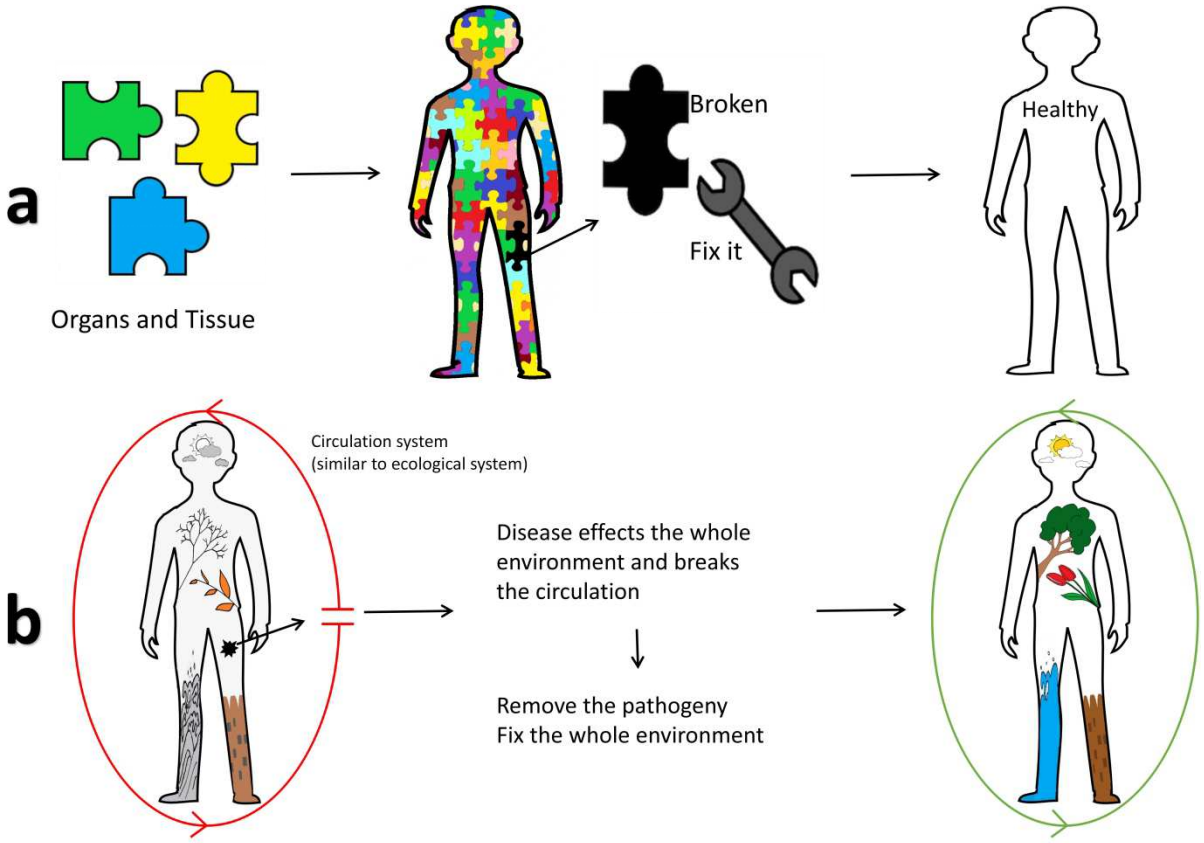


***Figure 1 Different philosophies between modern medicine and Traditional Chinese Medicine (TCM):*** *a) Modern medicine views the body as a whole of parts that function differently treating a single part of the body when something goes wrong; b) According to TCM theory, the human body is a complete system, like a natural ecosystem, with a normal circulation system. The human body forms a smooth circulation system, similar to the ecological system, including the circulation of "qi" (an abstract concept, the most basic substance that constitutes the human body and maintains its vital activities, it has the functions of pushing, warming, defense, fixation, gasification and nutrition), blood and "essence" (the internal humors and normal secretions of the organs, the generic name of all normal fluids of the body). When the disease occurs, the whole-body environment changes, so the cure for the disease is to adjust the whole system and restore circulation.*

## 2.2 TCM in oncotherapy

Cancer treatment using TCM has a long history in China and is now well integrated into current health care systems. The occurrence of a tumor is believed to be caused by the imbalance between the healthy body and the malignant factors: the body is either not enough strong, or the malignant factors are too potent. In TCM, treatment is based on the differentiation of syndromes. According to syndrome differentiation, TCM mainly emphasizes the treatment of "Yin and Yang balance"; the balance between "strengthening the body resistance to eliminate pathogenic factors", and "detoxifying and removing blood stasis" [34]. Based on the TCM theory, Chinese medicines

therefore used for treating cancer are divided into two main categories: "strengthening the body resistance" and "attacking pathogen", as shown in Figure 2.

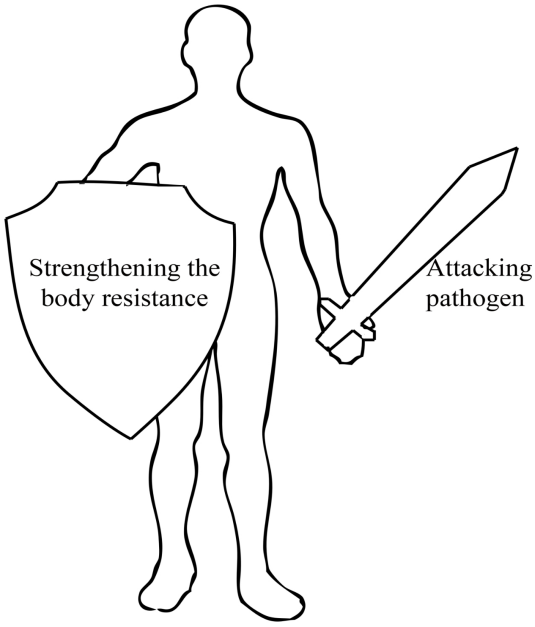


***Figure 2 Chinese herbs for oncotherapy classification according to the TCM theory:*** *Chinese medicines are divided into two main categories: "strengthening the body resistance" and "attacking pathogen" based on the TCM theory. Medicines used as "strengthening the body resistance" strengthen the human body, to make it like a shield against disease; Others used as "attacking pathogen" act as a weapon to help the body drive away disease.*

In clinical practice, TCM usually uses a combination of several medicines as a formula. Therefore, in most clinical investigations the prescriptions were compounds rather than single drugs. The good curative effect of Chinese medicine formulas has been verified in numerous clinical observations. In a multicenter, prospective cohort study [35], Yiqi Huayu Jiedu Decoction along with chemotherapy-treated 251 cases of gastric cancer patients (stages II and III), compared with 238 patients with chemotherapy alone, found that the group using a combination of TCM and chemotherapy had a lower risk of recurrence. There remains a wealth of evidence for the role of Chinese medicine formulas in Western biomedicine and TCM. The good curative effect of "strengthening the body resistance" medicines, when used as an auxiliary for chemotherapy, has been found in modern medicine to be mainly related to reducing toxicity, increasing efficiency and regulating immunity. The contribution of TCM in alleviating adverse reactions is unquestionable; Yiguan Decoction and Xiangsha Liujunzi Decoction combined with western medicine can relieve vomiting, anorexia, and fatigue adverse reactions caused by chemotherapy [36]. Regarding TCM

effects on regulating immunity, routine chemoradiotherapy combined with Yiqi Yangyin Decoction can effectively reduce tumor load and optimize the anti-tumor immune function in patients with advanced non-small-cell lung cancer [37]. Another clinical study found that Docetaxel-Cisplatin (DC) chemotherapy combined with Yiqi Guben Decoction in the treatment of advanced non-small-cell lung cancer, could effectively reduce the serum tumor marker levels, the inflammatory stress and improve immune function [38]. Given the outstanding performance of TCM in this area, some of the effective compounds have even been approved for mass production as adjuvant drugs for chemoradiotherapy, such as Shenqi Fuzheng Injection [39]. The most representative example of "attacking pathogen" medicine against the tumor is the remarkable efficacy of arsenic in the treatment of leukemia. Other representative TCM patent prescriptions of "attacking pathogen" in clinical observation, such as Aidi Injection, not only improves the efficacy of cisplatin regime but also has the function of reducing adverse events and preventing the overexpression of P-glycoprotein (P-gp) induced by chemotherapy of cisplatin regimen [40]. Recent studies suggest that treating advanced gastric cancer with sodium cantharidinate could improve the quality of life, decrease bone marrow suppression and gastrointestinal side-effects [41]. Xiaoaiping Injection as a retention enema in the treatment of colorectal cancer, can significantly improve the clinical efficacy and relieve clinical symptoms of patients [42].

### 2.3 TCM in colon cancer therapy

TCM theory believes that colon cancer is caused by internal and external factors together. It is caused by internal injury, dietary habits and emotional disorder [43]. The basic pathogenesis is the accumulation of dampness, heat and blood stasis in the intestinal canal and the loss of conduction [44]. Coupled with the "strengthening the body resistance" and "attacking pathogen" theory, the principle of clinical treatment is mainly to invigorate the spleen and replenish qi, and to nourish Yin and kidney. At the same time, different methods such as dispelling dampness, clearing heat, promoting blood circulation and detoxification are used according to syndrome differentiation [45]. Clinical application of TCM as supplement therapy of colon cancer showed that TCM has certain advantages in improving immunity, prolonging median survival, reducing adverse reactions, improving quality of life and other aspects [46].

After 5 years of follow-up, the recurrence rate, overall survival rate and median survival time of 60 patients with colorectal cancer treated with Tianma Granule + standard chemotherapy were significantly better than those in the control group; after one course of follow-up, the main immune indexes IgA, IgM, IgG and C3 of patients treated with Tianma granules were significantly better than those of the control group [47]. A study has observed the effects of TCM invigorating spleen

therapy on the balance of Th1 /Th2 immune response, cancer-induced fatigue and peripheral neuropathy in patients after colon cancer surgery: the incidence of neuropathy in the observation group was 61.34% which was significantly lower than that in the control group; IFN - γ, IL - 2 TNF - α and IL - 4 in the observation group were significantly better than that in the control group; in 1, 3 and 6 months after treatment, the score of cancer-related fatigue in the observation group were also significantly lower [48]. A study of 34 patients with advanced metastatic colorectal cancer by using Jianpi Xiaoai formula + FOLFIRI chemotherapy showed that the Jianpi Xiaoai formula + FOLFIRI chemotherapy could improve the disease control rate, prolong the progression-free survival and OS, reduce the hematic or gastrointestinal adverse reactions, and improve patients' quality of life [49]. Another meta-analysis of 14 randomized controlled trials suggested that compound Kushen injection combined with chemotherapeutic drugs was favorable for the treatment of advanced colon cancer and could improve the patients' life quality [50]. Xihuang pill containing serum can reverse the drug resistance of colon cancer drug-resistant cell lines HCT-8 /V and HCT-116 /L in vitro, which may be related to the down-regulation of the mRNA and protein expression levels of drug-resistant genes PKCα, Nrf2, MRPP1, MRP2 and GSTP1 [51].

In addition to oral drugs, there are also external treatments of TCM. External treatments of TCM for colon cancer take various forms, with enema being the majority of clinical applications, including acupuncture, external application and so on. TCM enema method is a kind of external therapy to treat diseases by injecting decoction into the anus or dropping it into the intestines. It is simple, easy to use, rapid-action, and not only can be used to treat local diseases but also for systemic diseases [52]. This method has the advantage of drugs acting directly on the tumor in colon cancer. Some enema formulations may suppress tumors, while others may relieve symptoms. For example, 50 patients with advanced colorectal cancer received chemotherapy combined with a TCM preparation called Qingchang Xiaoai granules enema treatment, the disease control rate was 78.0%, higher than the patients who received chemotherapy alone, and the incidence of diarrhea, vomiting, granulocytopenia was lower [53]. A decoction of *Magnolia officinalis*, *Rheum palmatum* and *Citrus aurantium*, was used as an enema to treat patients with enteric paralysis after a laparoscopic colon cancer operation, which achieved good clinical effect and could make patients' gastrointestinal function recover faster [54].

Basic studies in vivo and in vitro have shown that even single herbal extracts are effective against colon cancer. Tanshinone I, the active ingredient of *Radix salviae*, can induce apoptosis by upregulating Aurora A-p53 and mediate survival protein to play a significant inhibitory effect on the growth of colon cancer cells [55]. Baicalin, the major component of *Scutellaria baicalensis*,

could suppress a large number of oncomiRs, including miR-10a, miR-23a, miR-30c, miR-31, miR-151a and miR-205 by reducing the expression of c-Myc in colon cancer cells [56]. Ginsenoside Rg3, extracted from *Panax ginseng*, was found that repressed the growth and stemness of CRC cells both in vitro and in vivo and strengthened the cytotoxicity of 5-Fluorouracil and oxaliplatin against orthotopic xenografts in vivo [57]. Cinobufagin, isolated from the skin and posterior auricular glands of the *Asiatic toad*, could reverse multidrug resistance mediated by P-gp in colon cancer. After acting on LoVo/ADR, HCT116/ADR and CaO-2/ADR cell lines, it was found that cinobufagin enhanced the apoptosis of chemotherapy drugs and the sensitivity of drugs to P-gp overexpressed cells [58].

## 3 Mechanisms of traditional Chinese medication components in oncotherapy

At present, the mains recognized mechanisms of TCM in cancer treatment are: cytotoxicity, anti-metastasis, anti-angiogenesis, the reversal of MDR, inducing differentiation, regulating immunity, toxicity reducing and efficacy enhancing (for chemotherapy and radiotherapy).

### 3.1 Cytotoxicity

Many herbs used in TCM, like *Oldenlandia diffusa*, *Scutellaria barbata*, *Solanum lyratum*, *Paris polyphylla*, contain cytotoxic components. Their components mainly inhibit cell proliferation and induce apoptosis. For example, after spongioblastoma U87MG cells transformation induced by paclitaxel (from *Chinese yew*), the spindle appeared, the number of centrosomes changed and the nuclear crumbled [59]. Tripterine (from *Tripterygium wilfordii*) can activate DR-5/ p53/ Bax/ cysteine aspartic acid-specific protease (Caspase)-9/ Caspase-3 and DR-5/ FADD/ Caspase-8/ lysosomal/ cathepsin B/ Caspase-3 signaling pathway to inhibit osteosarcoma MG63 cells [60]. Saikosaponin-d (from *Bupleurum chinense*) can inhibit the proliferation of HeLa cells through blocking the cell cycle at the G1 phase and induce apoptosis [61].

### 3.2 Anti-metastasis

Even when the effects of chemotherapy and radiation are significant, many patients will lose their lives due to recurrence and metastasis. Research has found that geranyl lignin (from the lemon) through matrix metalloproteinases (MMP) pathway could inhibit the expression of MMP-2 and MMP-9 protein levels, reduce the hydrolysis of the extracellular matrix (ECM), and the ECM has powerful resistance to invasion and metastasis in liver cancer [62]. Norcantharidin (acantharidin derivative, from *Lytta vesicatoria*) inhibits the expression of TGF- beta and the Epithelial-Mesenchymal Transition (EMT) in hepatocellular carcinoma cells, by regulating TGF- beta /Smads signaling pathway that prevents the expression of EMT related transcription factors [63]. Berberine

(from *Coptis chinensis*) can significantly inhibit the extracorporeal and internal migration of HCT-116 cells in colorectal cancer by regulating the RAS-ERK signaling pathway [64].

### 3.3 Anti-angiogenic

There are currently many adverse reactions and contraindications of using anti-angiogenesis drugs in western medicine. More and more, clinicians are considering alternative TCM medicine options. Curcumin (from *Curcuma longa*) has been found to inhibit vascular shape by regulating the Vascular endothelial growth factor (VEGF)-VEGFR2 signaling pathway to improve the hematopoietic defect induced by VEGF [65]. Wogonin (from *Scutellaria baicalensis*), capable of inhibiting gastric cancer cells Y705 phosphorylation loci of STAT3 activation, indirectly plays an inhibitory role of the VEGF expression, and can directly prevent the angiogenesis induced by VEGF [66].

### 3.4 Reversing MDR

At present, there is no optimal solution to MDR. Evodiamine (from *Evodia rutaecarpa*) can inhibit the proliferation of oxaliplatin resistance colon cancer cells, promote apoptosis, inhibit the phosphorylation of NFκB, and then reverse the drug resistance [67]. Resveratrol (from *Polygonum cuspidatum*) can reverse the drug resistance of colorectal cancer cells by inhibiting EMT, activate NFκB, and enhancing colon cells' sensitivity to 5-Fu [68].

### 3.5 Inducing Differentiation

There is sparse research on the TCM mechanism of induction of cell differentiation, For example, shikonin (from *Lithospermum erythrorhizon*) and glycyrrhizin (from *Glycyrrhiza uralensis*) seem capable of inducing leukemia HL-60 cell differentiation [69, 70]. In the case of glycyrrhizin, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase plays an important role in this process [70].

### 3.6 Regulating immunity

Regulating immunity is a crucial aspect of TCM in the treatment of cancer. Soybean sterol B (from *Glycine max*) can significantly inhibit the growth and metastasis of subcutaneous tumors in mouse models of osteosarcoma and induce the immune response of mice against tumor [71]. Polysaccharides extracted from astragalus mongholicus and Codonopsis pilosula can effectively enhance the dendritic cell-based vaccine 4T1 preventing breast cancer metastasis in mice, can enhance the expression of CD40, CD80 and CD86 markers, and effectively modulate the specific cellular function of dendritic cells [72].

3.7 Reducing toxicity and enhancing efficacy

Adverse reactions of radiotherapy and chemotherapy seriously affect the quality of life of oncologic subjects. TCM can reduce toxicity and enhance the efficacy of chemotherapy and radiotherapy [73]. After observing 191 patients with advanced lung cancer treated with platinum-containing chemotherapy regimen along with or without Shengbai oral solution, it has been shown that the latter could decrease bone marrow suppression caused by chemotherapy in these patients, and reduce the amount of G-CSF used in these subjects [74]. However, at present, there is little research on the molecular mechanism underpinning TCM capability to reduce the toxicity of chemoradiotherapy and enhance the efficacy.

## 4 Solasonine and Solamargine

Solasonine and solamargine both are active ingredients extracted from *Solanum nigrum*. As shown in Figure 3, solasonin has the molecular formula $C_{45}H_{73}NO_{16}$ with a molecular weight of 884.06, and solamargine has the molecular formula $C_{45}H_{73}NO_{15}$ with a molecular weight of 868.06.

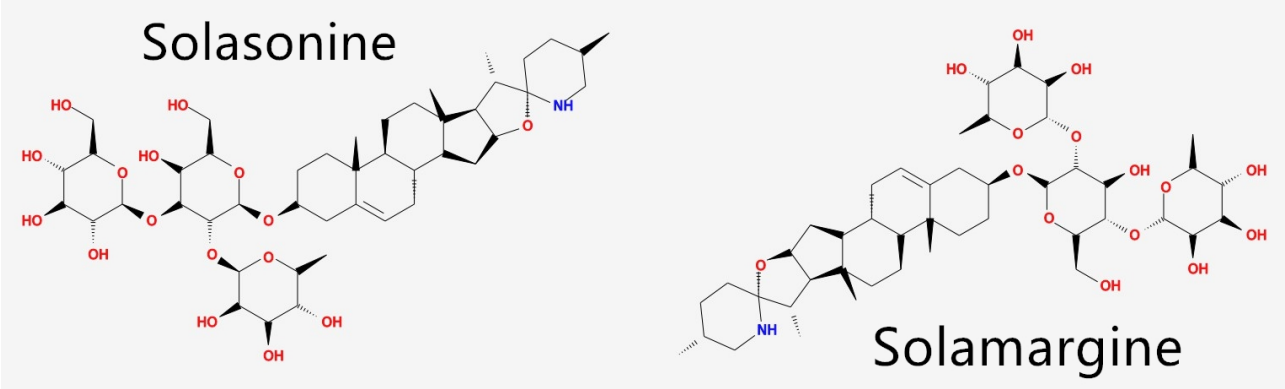


***Figure 3 The molecular structure of Solasonine and Solamargine***

4.1 The anti-cancer effect of *Solanum nigrum*

*Solanum nigrum* (Longkui) is an annual herb with a grass height of 30-120 cm: stems are erect and branched; leaves are ovate or heart-shaped; small white flowers in summer, 4-10 into a cyme; berries are spherical and dark purple when ripe. Both the berries and the leaves are edible, but the leaves contain a lot of alkaloids and must be cooked before detoxification. The whole plant can be used as medicine with the efficacy of removing blood stasis, detumescence and detoxification. In clinical application, *Solanum nigrum* is effective against a variety of tumors. Longkui Tingli Decoction, a TCM formula consisting of *Solanum nigrum* and seeds of *Draba nemorosa*, was used to treat lung cancer patients with massive pleural effusion. 35 lung cancer patients with large

pleural effusion who received Longkui Tingli Decoction + cisplatin therapy had improved immune function, longer median survival, and less severe symptoms compared to patients who only received cisplatin chemotherapy [75]. A TCM mixture mainly contains *Solanum nigrum*, called Longkui mixture could improve the clinical symptoms, liver function and immune function of patients with advanced primary liver cancer, effectively improve the quality of life of patients, and is expected to prolong the survival period [76]. Qifu Longkui decoction which mainly made of *Hedysarum multijugum*, *Aconitum carmichaeli*, *Solanum nigrum*, *Zingiberis rhizoma*, *Acoritataninowii rhizoma*, *Agrimonia eupatoria*, etc, showed good efficacy in metastatic colorectal cancer when combined with chemotherapy: After the treatment of 25 patients with Qifu Longkui Decoction combined with chemotherapy, the disease control rate was significantly better than that of the patients who only received chemotherapy, and the total incidence of leukopenia, nausea and vomiting, and alopecia were lower [77]. After the treatment of 48 patients with Qifu Longkui Decoction combined with FOLFOX chemotherapy, the tumor indexes CEA, LN, and CA19-9 decreased faster than the patients receiving FOLFOX chemotherapy alone, the immune indexes were also better than the control group, and the incidence of bone marrow suppression was lower [78].

In addition to clinical observation, mechanism studies also showed that *Solanum solanum* has a good anti-tumor effect. Qiyusanlong decoction composed of *Solanum nigrum*, *Astragalus membranaceus*, *Polygonatumod oratum*, scolopendra, pberetima, *Hedyotis diffusae*, *Coicis semen*, *Euphorbia helioscopia*, *Curcuma longa* and tendril-leaved *Fritillary bulb*, could repress lung tumor growth, modulate the expression of regulatory proteins in the Wnt/β-catenin pathway, including Wnt1, Wnt 2, Wnt 5a and glycogen synthase kinase 3β, ultimately suppress lung cancer [79]. *Solanum nigrum* could inhibit the growth rate of cervical cancer HeLa cells and increase the rate of apoptosis, and the strength of the effect is positively correlated with the time of intervention and the concentration [80]. The hydro-alcoholic extract of *Solanum solanum* was studied on normal Chinese hamster ovarian cells, rat fibroblast, and HepG2, CT26 cancer cell lines. It showed that the *Solanum nigrum* extract has cytotoxicity on cancer cells [81]. The total extract of *Solanum nigrum* had a cytotoxic effect on U266 myeloma cells, which could affect the cell cycle, decrease G0/G1 phase cells, increase S phase and $G_2$/M phase cells, and increase apoptosis cells [82]. The polyphenol-rich extract can inhibit the proliferation of human prostate cancer cells, stimulate cell apoptosis, and block the cell cycle in the $G_2$/M phase [83]. The extract of leaves had significant cytotoxic effects on human breast cancer AU565 cells: Low dose could induce autophagy but not apoptosis of tumor cells; high dose could inhibit the phosphorylation of Akt and induce autophagy and apoptosis of tumor cells [84].

Because of the significant anti-tumor effect of *Solanum nigrum* and the advantages of TCM enema, Professor Liu Shenlin from Jiangsu Provincial Hospital of Chinese Medicine summed up an enema medicine containing *Solanum nigrum*, *Agrimonia eupatoria* and *Herba patriniae* based on years of clinical experience. It showed that this enema combined with chemotherapy in the treatment of advanced colorectal cancer could improve the clinical objective efficacy and disease control rate, and reduce the level of serum VEGF in patients [85]: The patients were randomly divided into experimental group (n = 20) and control group (n = 20). The experimental group was treated with enema combined with FOLFOX chemotherapy, while the control group was treated with FOLFOX chemotherapy alone; after two cycles of treatment, the remission rate in the experimental group was 85%, higher than that in the control group (55%), and, the mean expression level of VEGF in the experimental group was 299.19ng/L, lower than that in the control group (344.24ng/L). To identify the active components, a liquid chromatograph-mass spectrometer (LC-MS) was used to detect the main components, the results showed that its aqueous extract was rich in solasonine (96.17 μg/mL) and solamargine (34.64 μg/mL) [86].

Based on this background, for identifying the active ingredients in this effective enema, exploring their mechanism of action, and developing new drugs and treatment methods, this study observed the effects of solasonine and solamargine on the proliferation of colon cancer cells HT-29, HCT116, and SW620, and explored the possible related signaling pathways.

4.2 Current anticancer studies on solasonine and solamargine

It has been reported that, treatment of cancer cells with solamargine or solasonine inhibits the growth of the cells in vitro as well as tumor growth in vivo [87].

Solasonine could inhibit cell proliferation, invasion, and metastasis of glioma cells also could induce apoptosis via modulating cytochrome c (Cyt-c) and Caspase signaling; meanwhile, it decreased the expression of proinflammatory mediators and nuclear translocalization of NF-κB p50/p65 and might target anti-inflammatory signaling pathway [88]. It could inhibit the gastric cancer cells' proliferation and the colony-forming potential along with inducing apoptosis, its anticancer effects may due to modulation of miR-486-5p which in turn exerted its molecular role by targeting PIK3R1 [89]. It also could inhibit hepatocellular carcinoma HepG2 cells' growth through the reciprocal regulation between the miR-375-3p and lncRNA CCAT1, and this might result in transcription factor SP1-mediated reduction of IRF5 expression [90]. Furthermore, solasonine increased lipid ROS levels in hepatocellular carcinoma HepG2 cells by suppression of glutathione peroxidase 4 (GPX4) and glutathione synthetase showing it could promote ferroptosis of HCC cells via GPX4-induced destruction of the glutathione redox system [91].

Solamargine could inhibit the viability of human cholangiocarcinoma QBC939 cells in a dose-dependent manner, also induce the apoptosis and alter the mitochondrial membrane potential; it decreased the mRNA level of Bcl-2, Bcl-extra-large and X-linked inhibitor of apoptosis protein but increased the mRNA level of Bcl-2-associated X protein and inhibited the expression of poly ADP ribose polymerase (PARP), while promoted the expression of cleaved PARP [92]. It inhibited the growth of metastatic and primary melanoma cells WM239 and WM115, with minimum effect on normal and benign WM35 cells; it also caused cellular necrosis to WM115 cells and WM239 cells by induction of lysosomal membrane permeabilization and disrupted the intrinsic apoptosis pathway as revealed by the downregulation of hILP/XIAP which resulted in Caspase-3 cleavage, upregulation of Bcl-xL and Bcl-2, and downregulation of Apaf-1 and Bax in WM115 and WM239 cells only [93]. It also could inhibit hepatoma SMMC7721 and HepG2 cells' proliferation and induce apoptosis, it caused cell cycle arrest at the G2/M phase; solamargine downregulated the expression levels of Ki67 and pcna and Bcl-2, and promoted the activity of Bax, Caspase-3, and Caspase-9 [94].

These studies revealed that both solasonine and solamargine have anticancer activity, and their anticancer effects mainly belong to cytotoxicity, including inhibition of tumor cell proliferation and induction of apoptosis. Although solasonine and solamargine showed inhibitory effects on a variety of tumors in the above studies, due to the background and original intention of our study, we aimed to understand the mechanism of action of the active ingredients in an effective TCM enema. As mentioned above in section 4.1, this enema is used in the treatment of colon cancer in TCM, so in this study, our direction is to explore the anti-colon cancer effect of solasonine and solamargine. Before this study, some preliminary experiments have been carried out in China, such as the MTT experiment and scratch experiment of SS and SM with a wide range of concentration gradient on colon cancer cells. Based on these preliminary experimental results, this study was conducted.

## 5 Apoptosis

Apoptosis plays an important role in the evolution of organisms, the stability of the internal environment, and the development of multiple systems. Different from cell necrosis, it's not a passive process, but an active process, which involves the activation, expression, and regulation of a series of genes. The process of cell apoptosis can be summarized in firstly receiving the apoptotic signal, causing the apoptotic regulation of the molecular interaction, and then the activation of a proteolytic enzyme, into a continuous reaction process. From the perspective of apoptosis, cancer can be considered as the result of inhibition of the apoptotic mechanism of cells and failure to carry out death clearance. A series of oncogenes and proto-oncogenes are activated and overexpressed in

cancer cells, many of them are also important regulatory factors of apoptosis. Overexpression of those oncogenes can block the apoptotic process and prevent cancer cell reduction. According to the different initiation modes, there are three main pathways of apoptosis: mitochondrial, endoplasmic reticulum, and death receptor pathways.

The mitochondrial pathway activates Caspase by releasing pro-apoptotic enzyme activators through mitochondria [95]: Bcl-2 family members containing the BH3 domain interacting with other Bcl-2 family members that bind to the outer membrane of mitochondria or exist in the cytoplasm, resulting in changes in mitochondrial membrane permeability, loss of transmembrane potential, and release of Cyt-c and other proteins; In the presence of deoxyadenosine triphosphate (dATP), Cyt-c released into the cytoplasm can bind to apoptosis-related factor 1 to form a polymer, and then collect the precursor of Caspase-9 in the cytoplasm, promoting Caspase-9 binding and the induction of apoptotic bodies formation; activated Caspase-9 triggers other Caspases activation such as Caspase-3 and Caspase-7, to induce apoptosis.

The apoptotic signal of the endoplasmic reticulum pathway is caused by abnormalities in the endoplasmic reticulum [96]: the alteration in endoplasmic reticulum $Ca^{2+}$ equilibrium or excessive accumulation of endoplasmic reticulum proteins induces the expression of Caspase-12 inside the endoplasmic reticulum and the transfer of cytoplasmic Caspase-7 to the surface of the endoplasmic reticulum. Caspase-7 can activate Caspase-12, while activated Caspase-12 can further activate Caspase-3 and induce apoptosis.

A variety of external factors are used as the initiators of apoptosis, and then the apoptosis signal is transmitted through different signal transmission systems to cause apoptosis. Death receptor is a class of transmembrane proteins belonging to the tumor necrosis factor receptor gene superfamily. The extracellular part contains a cysteine-rich region, and the cytoplasmic region contains a structure composed of homologous amino acid residues, which have a proteolytic function [97].

### 5.1 Apoptosis and Caspases

Caspases are a group of structurally related cysteine proteases found in the cytosol. An important common feature of them is that their active sites all contain cysteine and specifically peptide bond break behind the aspartic acid residue. Among Caspases, Caspase-2, Caspase-8, Caspase-9 and Caspase-10 are involved in the initiation of apoptosis. Caspase-3, Caspase-6 and Caspase-7 are involved in the execution of cell apoptosis. Caspase-3 and Caspase-7 have a similar substrate and inhibitor specificity, and they degrade PARP and DNA fragmentation factor-45 (DFF-45), leading to the inhibition of DNA repair and the initiation of DNA degradation. The substrates

of Caspase-6 are lamin A and keratin 18 which lead to the collapse of the nuclear fiber layer and cytoskeleton [98]. The Caspases synthesized in cells exist in an inactive proenzyme state and can perform their function after activation. It is believed that there is an upstream and downstream relationship between Caspase-2,8,9,10 (the initiators) and Caspase 3,6,7 (the practitioners) in apoptosis.

### 5.2 Apoptosis and Bcl-2

Bcl-2 gene is a kind of oncogene, which can significantly inhibit apoptosis. Many genes with high homology with Bcl-2 were found, which together constituted a large Bcl-2 family, some of them can inhibit apoptosis, while others can promote apoptosis [99]. Mammalian Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1 are anti-apoptotic proteins, while Bax, Bcl-Xs, Bad, Bak, Bik/Nbk, Bid and Harakiri are proapoptotic proteins. Bcl-2 inhibits cell death caused by a variety of cytotoxic factors; overexpression of Bcl-2 can enhance cell resistance to most cytotoxins. It can enhance the resistance of cells to most DNA damage factors and inhibit the apoptosis induced by most chemotherapy drugs, but it can not inhibit the damage of these factors to cells. Likewise, it does not promote DNA repair. The role of Bcl-2 may be to prevent the signal activating the apoptotic mechanism from reaching its target molecules after DNA damage. Sometimes, high levels of Bcl-2 may indicate that downstream apoptotic pathways are still functional and Bcl-2 overexpression even can be a marker of chemosensitivity and favorable prognosis in certain cancers [100]. In cytotoxic T cells, apoptosis induced by the activation of the Caspases family by granase B is independent of Bcl-2, and granase B may only act downstream of the Bcl-2 regulatory site in the apoptotic pathway.

### 5.3 Apoptosis and NFκB

The mammalian transcription factor NFκB family is composed of P50 (a product of P105, both known as NFκB1), P52 (a product of P100, both known as NFκB2), cREL, P65, and Rel-B. These proteins are dimerized to form functional NFκB. In unstimulated cells, most of the NFκB dimer exists in an inactive state by binding to one of three cytoplasmic NFκB inhibitors (IκBs) that are IκB-α, IκB-β and IκB-ε. Signals activate NFκB by degrading IκBs, and the activated NFκB then enters the nucleus and binds to DNA: IκBs is first catalyzed by phosphorylation of its two conserved serine residues by IκBs kinase (IKK); IκBs is then polyubiquitinated and degraded by protease under the catalysis of SCF-E3 ubiquitin enzyme complex; Activated NFκB is translocated into the nucleus to bind to its associated DNA segments to induce the transcription of target genes. NFκB signal transduction pathway can inhibit cell apoptosis through a variety of pathways, which largely includes the IAPS family, Bcl-2 family, TRAF family, JNK, FLIP, A20, GADD45β,

MnSOD, but the specific mechanism is not fully understood [101]. Interestingly, the NFκB pathway can also promote the death of some tumor cells under certain conditions, and it has been suggested that its suppression may induce the development of cutaneous squamous cell carcinoma [102]. Although there is some controversy, the role of the NFκB pathway in promoting cell survival, promoting cell proliferation, and inhibiting cell death is generally accepted, inhibiting the activation of the NFκB signal transduction pathway to promote cell apoptosis, become a new way to treat tumors and other diseases [103].

# Materials and Methods

## 1 Cells

Colon cancer cell lines with different degrees of differentiation were selected. HT-29 cells (High differentiation), SW620 cells (Middle differentiation), HCT116 cells (Low differentiation), shared by Candiolo Cancer Institute FPO-IRCCS, Turin, Italy.

## 2 Solasonine and Solamargine

Solasonine: Solasonine powder was purchased from Nanjing jingzhu bio-technology Co., Ltd. 20mg white powder dissolved in 120μl dimethyl sulfoxide (DMSO) as mother solution. According to the results of the pre-experiment, the mother solution was diluted into 10μM, 15μM, 20μM, 25μM, 30μM, 35μM, 40μM with the medium. Concentrations of 10μM, 15μM, 20μM, 25μM, 30μM were used for HT-29 cells, and concentrations of 20μM, 25μM, 30μM, 35μM, 40μM were used for SW620 cells and HCT116 cells.

Solamargine: Solamargine powder was purchased from Nanjing jingzhu bio-technology Co. , Ltd. 20mg white powder dissolved in 120μl DMSO as mother solution. According to the results of the pre-experiment, the mother solution was diluted into 1μM, 2μM, 3μM, 4μM, 5μM, 6μM with the medium. Concentrations of 2μM, 3μM, 4μM, 5μM, 6μM were used for HT-29 cells, and concentrations of 1μM, 2μM, 3μM, 4μM, 5μM were used for SW620 cells and HCT116 cells.

## 3 Materials

RPMI 1640 medium containing 10% Fetal bovine serum (FBS), both were purchased from Corning Inc.. Dulbecco's phosphate-buffered saline (DPBS) was purchased from Corning Inc.. Thiazolyl blue tetrazolium bromide (MTT) and 5-Fu were purchased from Sigma-Aldrich LLC.. Tissue culture flasks, 96-wells tissue culture plates, 6-wells tissue culture plates, tissue culture dishes were purchased from VWR International LLC.. Microplate reader, "Multiskan Go" model, was purchased from Thermo Fisher Scientific Inc.. Hoechst 33342 was purchased from Thermo Fisher Scientific Inc.. Antifade mounting medium was purchased from Vector Laboratories Inc.. pSIVA-IANBD/ Propidium iodide (PI) kit was purchased from Abcam plc.. Fluorescence microscope was purchased from Nikon Corporation. Blocking buffer was purchased from Santa Cruz Biotechnology Inc.. Bradford Reagent, Monoclonal anti-β-Tubulin I and Tween 20 were purchased from Sigma-Aldrich LLC.. NE-PER nuclear and cytoplasmic extraction reagents kit, 8% Tris-Glycine gels, MOPS SDS running buffer, ECL western blotting substrate and LDS sample

buffer were purchased from Thermo Fisher Scientific Inc.. NFκB P65 antibody, p-NFκB P65 antibody, Caspase-3,9 antibodies and histone H2A antibody were purchased from Santa Cruz Biotechnology Inc.. B-cell lymphoma-2 (Bcl-2) antibody and IκB-α antibody were purchased from Cell Signaling Technology, Inc.. p-IκB-α antibody was purchased from Sigma-Aldrich LLC.. Gel imaging system (UVITEC) was purchased from Uvitec Ltd.. Bovin serum albumin (BSA) was purchased from Santa Cruz Biotechnology Inc.. Hydrogen peroxide solution, glycerol gelatin and 3,3'-Diaminobenzidine tetrahydrochloride were purchased from Sigma-Aldrich LLC.. Labelled polymer-HRP was purchased from Dako North America Inc.. NFκB P65 antibody and p-NFκB P65 antibody were purchased from Santa Cruz Biotechnology Inc.. Microscope camera system was purchased from Nikon Corporation.

**4 MTT Assay**

MTT assay is a method to detect cell survival and proliferation. The principle is that succinate dehydrogenase in mitochondria of living cells can reduce exogenous MTT to water-insoluble blue-purple crystal formazan and deposit it in cells, while it can not in dead cells. After DMSO dissolved the formazan, the light absorption of DMSO can be measured at 490nm with the microplate reader, which can indirectly reflect the number of living cells.

4.1 Groups

MTT assay was used to determine the effect of solasonine and solamargine on the proliferation of colon cancer cells. 5-Fu is the most widely used chemotherapeutic drug against colon cancer in clinic, so it was used as the positive control drug in MTT assay. According to the results of the pre-experiment, the groups were set as follows.

For solasonine:

HT-29 cells: Control group (medium), 10μM group, 15μM group, 20μM group, 25μM group, 30μM group and 5-Fu group (positive control, medium contain 5μg/ml 5-Fu);

SW620 cells: Control group (medium), 20μM group, 25μM group, 30μM group, 35μM group, 40μM group and 5-Fu group (positive control, medium contain 5μg/ml 5-Fu);

HCT116 cells: Control group (medium), 20μM group, 25μM group, 30μM group, 35μM group, 40μM group and 5-Fu group (positive control, medium contain 5μg/ml 5-Fu).

For solamargine:

HT-29 cells: Control group (medium), 2μM group, 3μM group, 4μM group, 5μM group, 6μM group and 5-Fu group (positive control, medium contain 5μg/ml 5-Fu);

SW620 cells: Control group (medium), 1μM group, 2μM group, 3μM group, 4μM group, 5μM group and 5-Fu group (positive control, medium contain 5μg/ml 5-Fu);

HCT116 cells: Control group (medium), 1μM group, 2μM group, 3μM group, 4μM group, 5μM group and 5-Fu group (positive control, medium contain 5μg/ml 5-Fu).

4.2 Procedure

1) 96-well plates were planted with 2000 logarithmic growing cells in each well, incubated overnight in the incubator with 37℃ and 5% $CO_2$.

2) After the cells adhered to the bottom of the wells, the culture medium was carefully sucked away, and the medium containing the corresponding drug concentration was added again according to different groups.

3) After 24 or 48 hours, the culture medium was carefully sucked away, medium containing 10% MTT solution (5mg/ml in DPBS) was added and incubated in the incubator for 4 hours.

4) The 96-Well plates were taken out and the culture medium containing MTT was carefully discarded. Then 150 μl of DMSO was added to each well and placed in the microplate reader. The OD value was read after 5 minutes of vibration.

5) Repeated 3 times.

**5 Hoechst 33342 Staining Assay**

Hoechst can cross the cell membrane and bind to the DNA of living or immobilized cells. When cells undergo apoptosis, chromatin shrinks. Therefore, after Hoechst 33342 staining, the nuclei of normal cells were normal blue under a fluorescence microscope, while the nuclei of apoptotic cells were dense and concentrated, or fragmentation dense and concentrated.

5.1 Groups

Hoechst 33342 staining was used to observe the nucleus damage rate, and according to the results of MTT, the groups were set as follows.

For solasonine:

HT-29 cells: Control group (medium), 15μMgroup and 30μM group;

SW620 cells: Control group (medium), 25μM group and 35μM group;

HCT116 cells: Control group (medium), 25μM group and 35μM group.

For solamargine:

HT-29 cells: Control group (medium), 3μM group and 4μM group;

SW620 cells: Control group (medium), 2μM group and 3μM group;

HCT116 cells: Control group (medium), 2μM group and 4μM group.

5.2 Procedure

1) Cover glasses were sterilized and placed in 6-well plates. Then the 6-well plates were planted with $5.0\times 10^4$ logarithmic growing cells in each well, incubated overnight in the incubator with 37℃ and 5% $CO_2$.

2) After the cells adhered to the cover glasses, the culture medium was carefully sucked away, and the medium containing the corresponding drug concentration was added again according to different groups.

3) After 24 or 48 hours, the culture medium was carefully sucked away. Each well was fixed with a few drops of paraformaldehyde for 15min, then sucked away, and paraformaldehyde was added again for further fixation for 15min.

4) Each well was gently rinsed 3 times with PBS.

5) 0.1% Hoechst 33342 was added to each well and then incubated at room temperature, away from light, for 3 minutes.

6) Each well was gently rinsed 3 times with PBS.

7) Clean slides were labeled and numbered, and a drop of the antifade mounting medium was applied to the slides, which were then covered with the cover glasses containing cells.

8) The slides were observed under the fluorescence microscope.

9) Repeated 3 times.

**6 pSIVA-IANBD/ PI Staining Assay**

The surface damage of the cell membrane occurs in the early stage of apoptosis. When the cell membrane is damaged, the phosphatidylserine on the surface of apoptotic cells can be flipped from the inner membrane to the outer membrane of the cell. pSIVA-IANBD can only produce green fluorescence detected in FITC channel after binding with everted phosphatidylserine, while unbound pSIVA-IANBD does not produce fluorescence so that the apoptotic cells can be detected when mixed with PI staining. When the cells fluoresce only in green, the cells are in the early stage

of apoptosis. When the cells fluoresce in green and red, the cells are in the late stage of apoptosis or non-apoptotic death.

6.1 Groups

pSIVA-IANBD/ PI staining was used to observe the apoptosis, and the groups were set as same as Hoechst 33342 staining assay.

6.2 Procedure

1) Cover glasses were sterilized and placed in 12-well plates. Then the 12-well plates were planted with $1.6\times 10^4$ logarithmic growing cells in each well, incubated overnight in the incubator with 37℃ and 5% $CO_2$.

2) After the cells adhered to the cover glasses, the culture medium was carefully sucked away, and the medium containing the corresponding drug concentration was added again according to different groups.

3) After 24 or 48 hours, 10μl pSIVA-IANBD and 5μl PI were added directly to each well and then incubated in the incubator with 37℃ and 5% $CO_2$, for 10 minutes.

4) Clean slides were labeled and numbered, and a drop of the antifade mounting medium was applied to the slides, which were then covered with the cover glasses containing cells.

5) The slides were observed under the fluorescence microscope.

6) Repeated 3 times.

**7 Western Blot Assay**

7.1 Groups

Western blot assay was used to detect the protein expression, and the groups were set as same as Hoechst 33342 staining assay.

7.2 Procedure

1) $5.0\times 10^5$ logarithmic growing cells were seeded in dishes, incubated overnight in the incubator with 37℃ and 5% $CO_2$.

2) After the cells adhered to the bottom of the dishes, the culture medium was carefully sucked away, and the medium containing the corresponding drug concentration was added again according to different groups.

3) After 24 or 48 hours, the NE-PER nuclear and cytoplasmic extraction reagents kit was used to extract nucleus protein and cytoplasm protein from cells in each dish.

4) The Bradford Reagent was used to measure protein concentration.

5) 20μg of protein was taken from each group, and all proteins were adjusted to the same concentration with $dH_2O$, the final volume was kept 15μl, 5μl LDS 4× was added, and then heated to 70℃ for 10min.

6) After the Tris-Glycine gel was fixed to the electrophoresis apparatus, 20μl protein prepared in Step 5) was added to each well. After the MOPS SDS running buffer was filled into the electrophoresis apparatus, the cover was put on and the power supply was adjusted to 100V 74mA for 90min.

7) The transfer solution 10× was prepared with 14.4g glycine and 3.03g Tris per 100ml, and the pH was adjusted to 8.3. Each 300ml transfer solution was prepared by 30ml transfer solution 10×, 60ml methanol and 210ml $dH_2O$.

8) A sandwich was made of the running gel, four pieces of sponges, two pieces of filter paper and a piece of PVDF membrane. The sandwich was placed in the transfer instrument, and the power supply was adjusted to 36 V, 110-170mA for 90min after pouring the transfer solution.

9) The PVDF membrane was taken out and immersed in the blocking buffer and placed on a shaker for 1h.

10) 500ml TBS 10× was prepared with 15g Tris, 40.03g NaCl and 1g KCl. The pH value was adjusted to 7.6. 500ml TBST buffer was prepared by 50ml TBS 10×, 449.5ml $dH_2O$ and 0.5ml Tween 20.

11) After blocking, the PVDF membrane was washed by TBST for 5s, then was immersed in the antibody solution for overnight at 4 ℃.

12) The membrane was washed 4 times with TBST on the shaker, 10min each time. Then it was immersed in the second antibody solution for 90min at room temperature.

13) The membrane was washed 4 times with TBST on the shaker, 10min each time. Then with the ECL western blotting substrate, it was read by the gel imaging system.

## 8 Immunocytochemistry Assay

8.1 Groups

Immunocytochemistry method was used to observe the phosphorylation and nucleus translocation of NFκB P65, and the groups were set as same as Hoechst 33342 staining assay.

8.2 Procedure

1) Cover glasses were sterilized and placed in 6-well plates. Then the 6-well plates were planted with $5.0\times 10^4$ logarithmic growing cells in each well, incubated overnight in the incubator with 37℃ and 5% $CO_2$.

2) After the cells adhered to the cover glasses, the culture medium was carefully sucked away, and the medium containing the corresponding drug concentration was added again according to different groups.

3) After 24 or 48 hours, the culture medium was carefully sucked away. Each well was fixed with a few drops of paraformaldehyde for 15min, then sucked away, and paraformaldehyde was added again for further fixation for 15min.

4) Each well was gently rinsed 3 times with PBS. Then each well was soaked with 0.1% Triton-X 100 solution for 10min.

5) Each well was gently rinsed 3 times with PBS again. Then each well was blocked with 3% BSA solution for 30min.

6) The BSA solution was carefully sucked away, then the cover glasses were immersed in the antibody solution overnight at 4 ℃.

7) After incubating the antibody, each well was gently rinsed 3 times with PBS, then the Labelled polymer-HRP was dropped on the cover glasses for incubating 35min at room temperature.

8) Each well was gently rinsed 3 times with PBS again. Then the cover glasses were immersed in the 3,3'-Diaminobenzidine tetrahydrochloride solution for 5min.

9) Each well was thoroughly washed with water. Then the cover glasses were stained with hematoxylin at room temperature for 1 min.

10) After washed with $dH_2O$, the cover glasses were sealed with glycerol gelatine over the labeled slide. Then slides were observed under the microscope camera system.

11) Repeated 3 times.

## 9 Statistics

The proliferation inhibition rate of each group was calculated by the following formula: Proliferation inhibition rate= (1- OD value / OD value of control group) ×100%.

Image J 1.8.0 software was used to count cell numbers, calculate the positive area and measure the gray value of the band.

SPSS 18.0 software was used for statistical analysis, and the One-way ANOVA test was used for comparison between multiple groups. $P< 0.05$ was considered statistically significant.

# Results

## 1 Inhibition of solasonine on the proliferation of colon cancer cells

As shown in Figure 4:

24 hours after solasonine treatment on HT-29, SW620 and HCT116 cell lines, different from the positive control group (i.e., 5-Fu group), the OD value of each concentration group was similar to that of the untreated control group (P> 0.05), so the proliferation inhibition rate could not be calculated. Considering the insufficient action time, the administration time was extended to 48 hours.

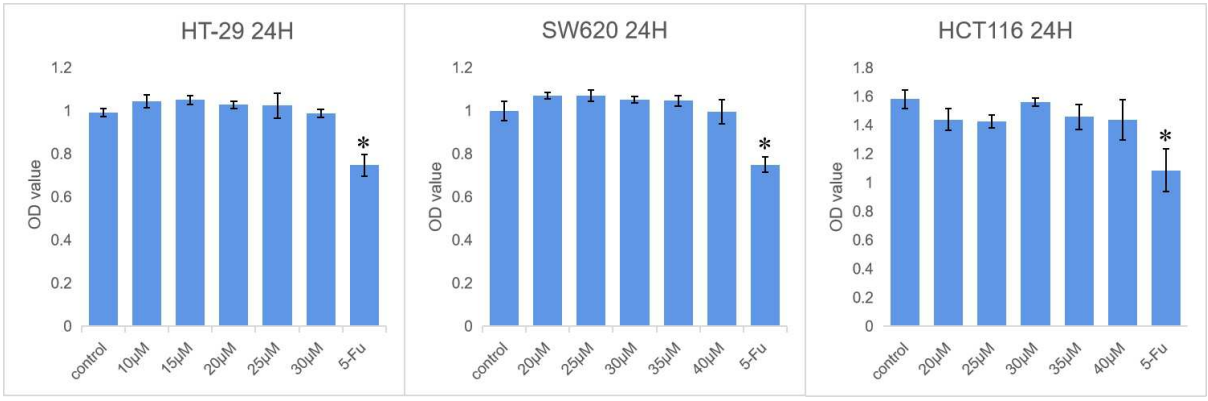


***Figure 4 OD values of each group after treated with solasonine for 24h:*** *significant differences were detected only for cells treated with 5-Fu (* P< 0.05 vs control group).*

As shown in Figure 5:

- The inhibition rates of HT-29 cell proliferation were (9.23 ± 2.86)%, (37.24 ± 1.91)%, (54.76 ± 1.21)%, (62.20 ± 2.19)% and (70.38 ± 0.49)%, respectively, after the treatment with different concentrations of solasonine for 48h; the comparison between groups was statistically significant (P< 0.05), and the inhibition rate increased with the increase of solasonine concentration.

- The inhibition rates of SW620 cell proliferation were (18.61 ± 1.45)%, (42.15 ± 2.69)%, (54.83 ± 1.82)%, (65.51 ± 1.24)% and (76.18 ± 2.15)%, respectively, after the treatment with different concentrations of solasonine for 48h; the comparison between groups was statistically significant (P< 0.05), and the inhibition rate increased with the increase of solasonine concentration.

- The inhibition rates of HCT116 cell proliferation were (11.14 ± 1.97)%, (31.31 ± 5.89)%, (61.17 ± 6.09)%, (70.24 ± 4.40)% and (76.42 ± 1.97)%, respectively, after the treatment

with different concentrations of solasonine for 48h; there were significant differences between 20μM group, 25μM group and 30μM group ($P< 0.05$), and no significant differences between 35μM group and 40μM group, but significant differences were found between 35μM group or 40μM group with other groups ($P< 0.05$). The inhibition rate increased with the increase of solasonine concentration.

According to the results above, we pick the concentration of solasonine as 15μM and 30μM for HT-29 cells, 25μM and 35μM for SW620 cells and HCT116 cells in next experiments, and the action time should be 48 hours.

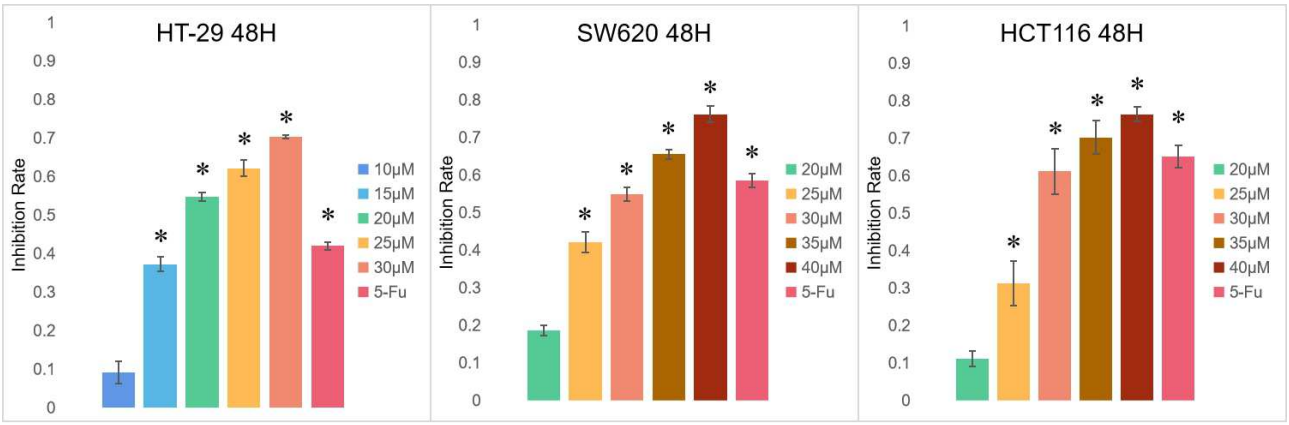


***Figure 5 Inhibition rates of solasonine on the proliferation of colon cancer cells:*** *After the treatment with solasonine for 48h, the MTT assay was performed, and the inhibition rate of each group was calculated (* $p< 0.05$ vs control group).*

**2 Solasonine induced apoptosis in colon cancer cells**

As shown in Figure 6:

Microscopically, the nuclei of the untreated cells (control group) were uniformly fluorescent and regular in shape; in the cells treated with solasonine, there were scattered nuclei wrinkled with enhancing fluorescence, even several fluorescence fragments as well as scattered nuclei with dimming fluorescence.

The nucleus damage rate was then measured:

- The nucleus damage rate of HT-29 control group was (1.54 ± 1.33)%, while that of 15μM group and 30μM group were (11.35 ± 1.55)% and (19.84 ± 4.03)% respectively; the comparison between groups was statistically significant ($P< 0.05$), and the nucleus damage rate increased with the increase of solasonine concentration.

- The nucleus damage rate of SW620 cell control group was (1.19 ± 0.40)%, while that of 25μM group and 35μM group were (6.85 ± 0.47)% and (17.68 ± 0.55)% respectively; the comparison between groups was statistically significant (P< 0.05), and the nucleus damage rate increased with the increase of solasonine concentration.
- The nucleus damage rate of HCT116 cell control group was (0.90 ± 0.79)%, while that of 25μM group and 35μM group were (9.35 ± 1.52)% and (15.23 ± 2.48)% respectively; the comparison between groups was statistically significant (P< 0.05), and the nucleus damage rate increased with the increase of solasonine concentration.

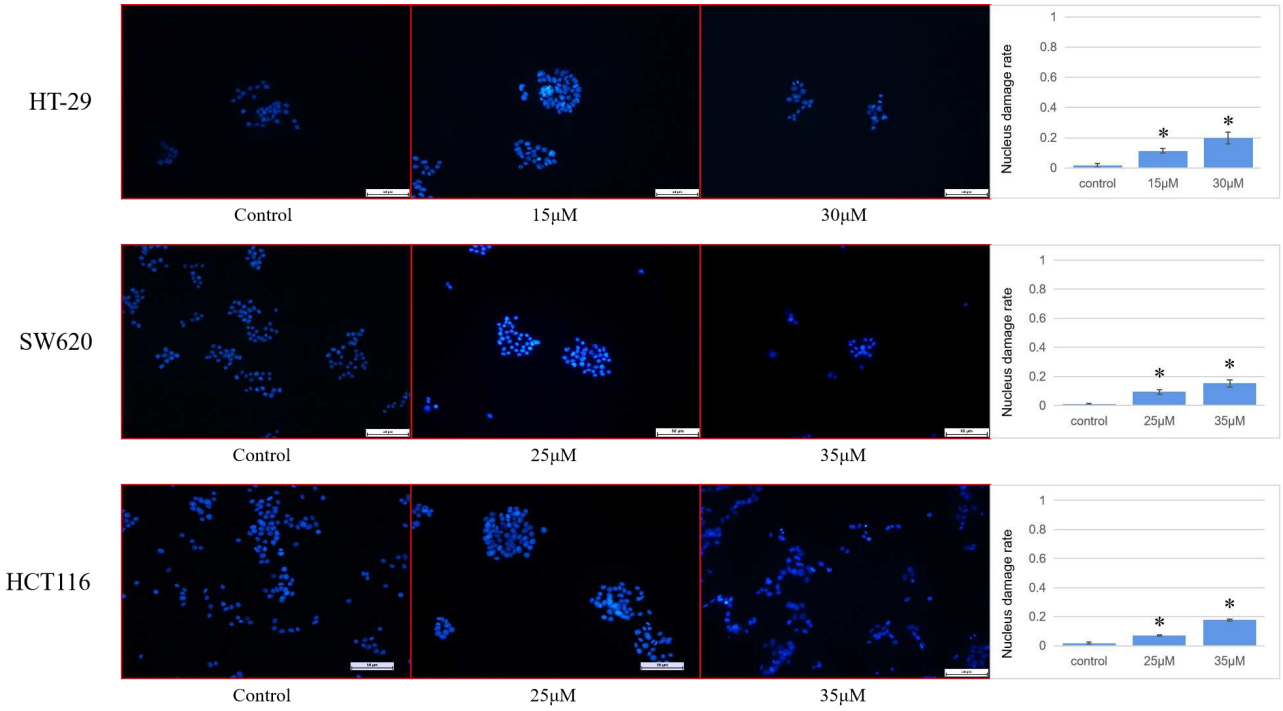


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***Figure 6 Nucleus damage rate of colon cancer cells after solasonine treatment (400× magnification):*** *After solasonine treatment for 48h, the nucleus damage rate of each group was measured (Hoechst* 33342 *staining) with fluorescence microscope assay. Histograms depict significant differences (* p< 0.05 vs control group).*

As depicted in Figure 7:

- In the HT-29 control group, pSIVA-IANBD positive areas accounted for (0.02 ± 0.01)% of the field of vision, while that in 15μM group and 30μM group accounted for (0.78 ± 0.03)%

and (1.12 ± 0.16)% respectively; In the control group, PI-positive areas accounted for (0.02 ± 0.004)% of the field of vision, while that in 15μM group and 30μM group accounted for (0.25 ± 0.05)% and (0.59 ± 0.09)% respectively; in both stainings, the comparison between groups was statistically significant ($P< 0.05$), and the positive areas increased with the increase of solasonine concentration.

- In the SW620 control group, pSIVA-IANBD positive areas accounted for (0.02 ± 0.01)% of the field of vision, while that in 25μM group and 35μM group accounted for (0.52 ± 0.10)% and (0.81 ± 0.08)% respectively; In the control group, PI-positive areas accounted for (0.02 ± 0.004)% of the field of vision, while that in 25μM group and 35μM group accounted for (0.23 ± 0.03)% and (0.31 ± 0.04)% respectively; in both stainings, the comparison between groups was statistically significant ($P< 0.05$), and the positive areas increased with the increase of solasonine concentration.
- In the HCT116 control group, pSIVA-IANBD positive areas accounted for (0.03 ± 0.01)% of the field of vision, while that in 25μM group and 35μM group accounted for (0.15 ± 0.04)% and (0.43 ± 0.04)% respectively; In the control group, PI-positive areas accounted for (0.04 ± 0.02)% of the field of vision, while that in 25μM group and 35μM group accounted for (0.06 ± 0.02)% and (0.10 ± 0.07)% respectively; in pSIVA-IANBD staining, the comparison between groups was statistically significant ($P< 0.05$), and the positive areas increased with the increase of solasonine concentration; in PI staining, there was no difference between the groups ($P> 0.05$).

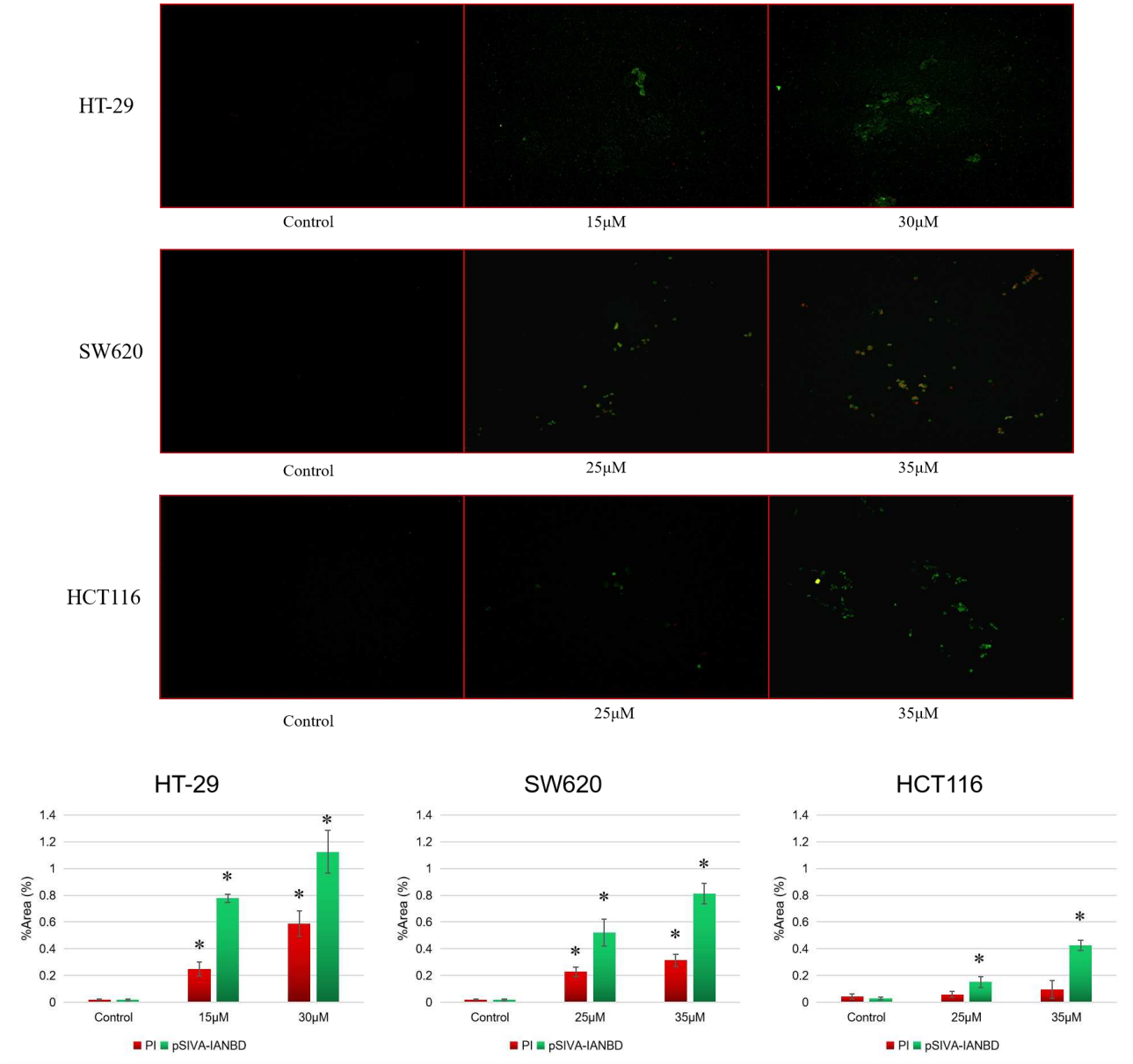


***Figure 7 The pSIVA-IANBD/ PI staining of colon cancer cells after solasonine treatment (200× magnification):*** *After different cells were treated with solasonine for 48h, the pSIVA-IANBD/ PI staining assay was performed. And after observed under the fluorescence microscope, the positive area rate of each group was counted (* P< 0.05 vs control group).*

## 3 Expression of apoptosis-related proteins in colon cancer cells after solasonine treated

As shown in Figure 8:

After 48 hours of treatment, the expression levels of IκB-α, p-IκB-α, pro-Caspase-3 and pro-Caspase-9 in HT-29 cells increased, while p-NFκB P65 in the cytoplasm and NFκB P65 in nucleus decreased in the 30μM group; the expression of IκB-α, p-IκB-α, pro-Caspase-3, pro-Caspase-9, p-NFκB P65, and NFκB P65 in the nucleus of the control group and 15μM group were similar; the

expression of bcl-2 in 15μM group decreased, while that of the control group and 30μM group were similar.

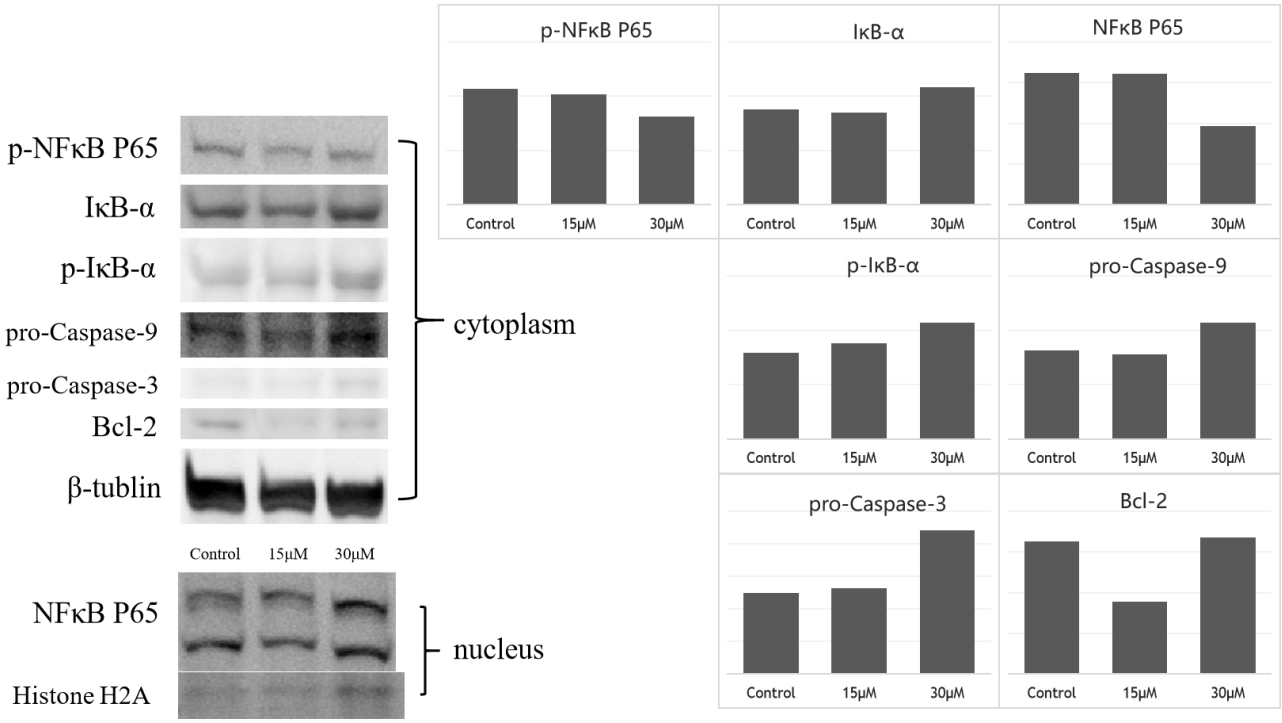


***Figure 8 Expression of apoptosis-related proteins in HT-29 cells after solasonine treatment:*** *After HT-29 cells were treated with solasonine for 48h, the Western blot assay was performed. And after reading by the gel imaging system, the gray value of each band was measured.*

As shown in Figure 9:

After treated for 48 hours, the expression levels of IκB-α, pro-Caspase-3 and pro-Caspase-9 in SW620 cells increased in the 25μM group and that of the control group and 35μM group were similar. NFκB P65 in nucleus decreased in 35μM group; the expression of p-IκB-α and p-NFκB P65 decreased as the concentration increased; the expression of Bcl-2 in 25μM group and 35μM group increased compared to the control group.

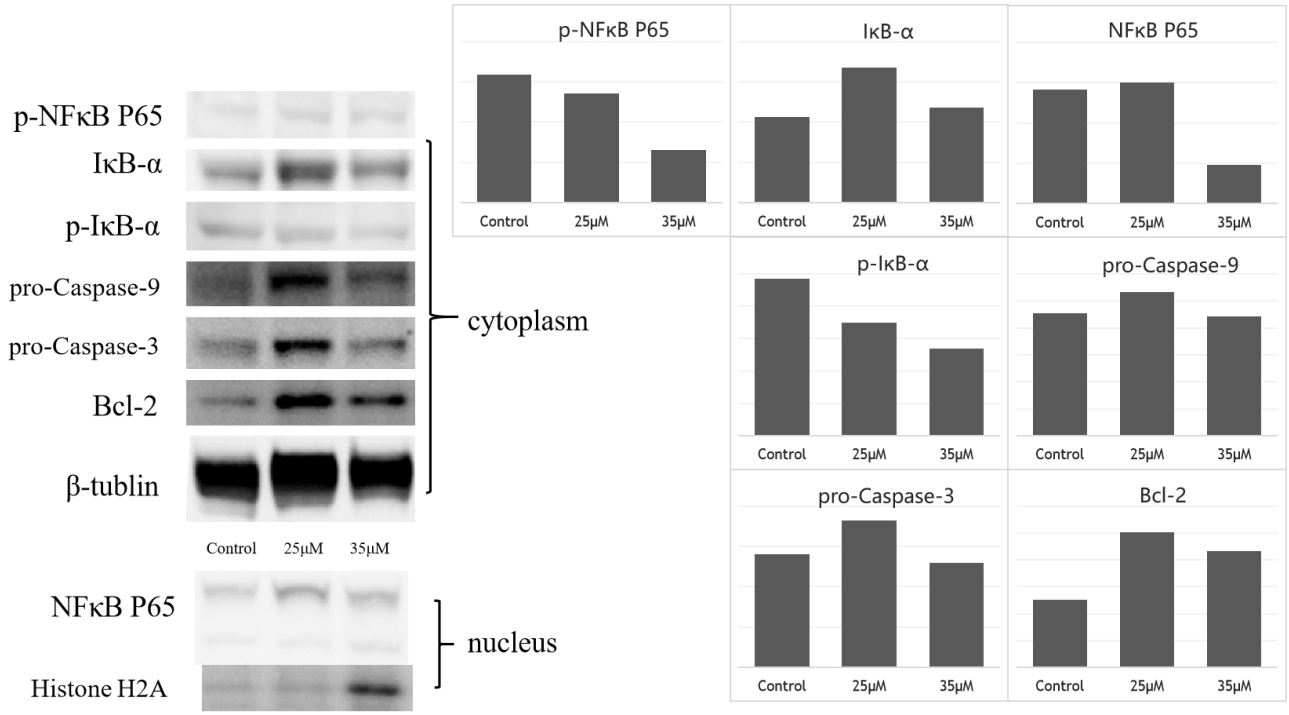


***Figure 9 Expression of apoptosis-related proteins in SW620 cells after solasonine treatment:*** *After SW620 cells were treated with solasonine for 48h, the Western blot assay was performed. And after reading by the gel imaging system, the gray value of each band was measured.*

As shown in Figure 10:

After 48 hours of treatment, the expression levels of IκB-α, Bcl-2, pro-Caspase-3 and pro-Caspase-9 in HCT116 cells increased both in 25μM and 35μM groups, while p-NFκB P65 in the cytoplasm and NFκB P65 in nucleus decreased; the expression of p-IκB-α decreased as the concentration increased.

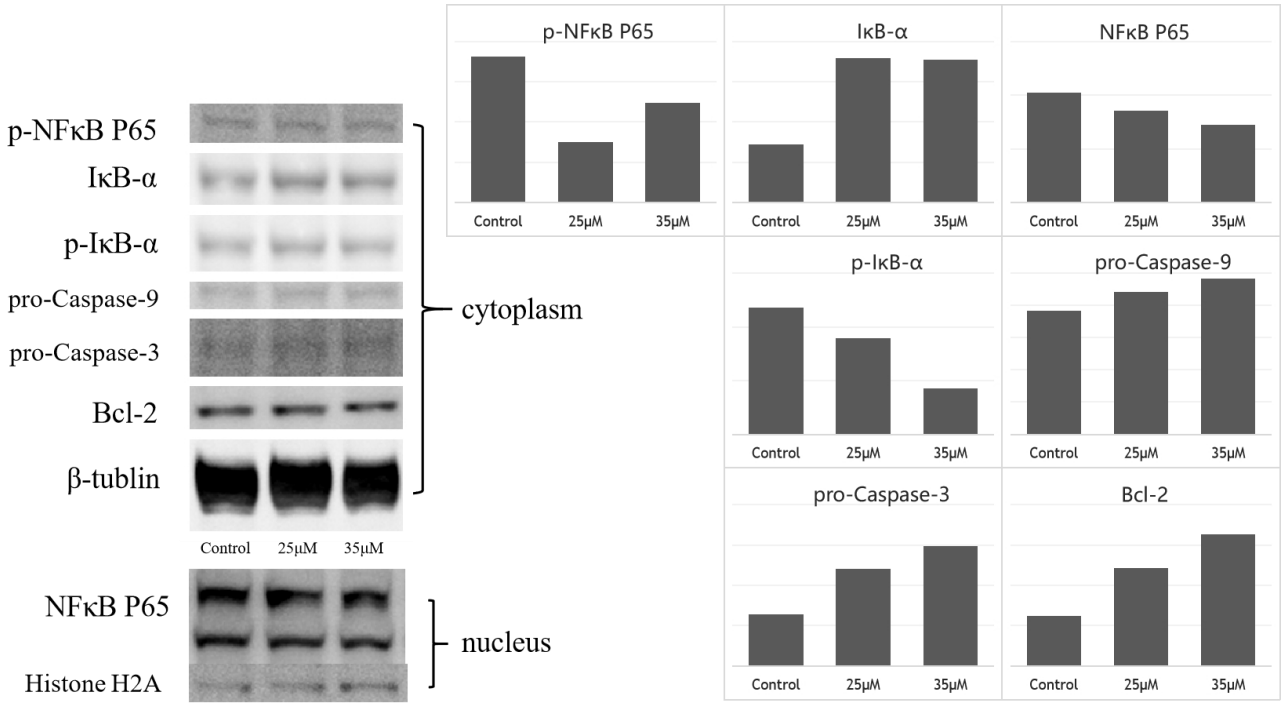


***Figure 10 Expression of apoptosis-related proteins in HCT116 cells after solasonine treatment:*** *After HCT116 cells were treated with solasonine for 48h, the Western blot assay was performed. And after reading by the gel imaging system, the gray value of each band was measured.*

The Western blot results showed that after solasonine treatment for 48h, the expression of pro-Caspase-3 and pro-Caspase-9 increased suggesting that these enzymes are not involved in the mechanism of apoptosis. Furthermore, the expression of anti-apoptotic protein Bcl-2 even showed different trends in different cell lines. However, these three cell lines all showed common and stable characteristics after solasonine treatment: p-NFκB P65 and p-IκB-α in cytoplasmic proteins, a decrease in nuclear NFκB P65, and an increase of cytoplasmatic IκB-α. According to these results, immunocytochemistry was used to observe the effect of solasonine on the NFκB signaling pathway in colon cancer cells.

**4 The effect of solasonine on the NFκB signaling pathway**

As shown in Figure 11:

- The proportion of HT-29 cells with high expression of NFκB P65 protein in the cytoplasm was (95.63 ± 4.18)% in the control group, while that of 15μM group and 30μM group were (76.50 ± 6.51)% and (65.42 ± 8.99)% respectively, and there was a statistical difference between groups ($P < 0.05$).
- The amount of HT-29 cells with no expression of NFκB P65 protein in the cytoplasm was (0 ± 0)% in the control group, while that of 15μM group and 30μM group were (1.05 ± 1.70)%

and (11.73 ± 5.12)% respectively, and there was a statistical difference between the control group and 30μM group (P< 0.05).

- The percentage of HT-29 cells with high expression of NFκB P65 protein in the nucleus was (77.82 ± 2.21)% in the control group, while that of 15μM group and 30μM group were (62.96 ± 6.69)% and (50.80 ± 6.72)% respectively, and there was a statistical difference between groups (P< 0.05).
- The proportion of HT-29 cells with no expression of NFκB P65 protein in the nucleus was (0 ± 0)% in the control group, while that of 15μM group and 30μM group were (8.41 ± 1.91)% and (18.69 ± 6.73)% respectively, and there was a statistical difference between groups (P< 0.05).

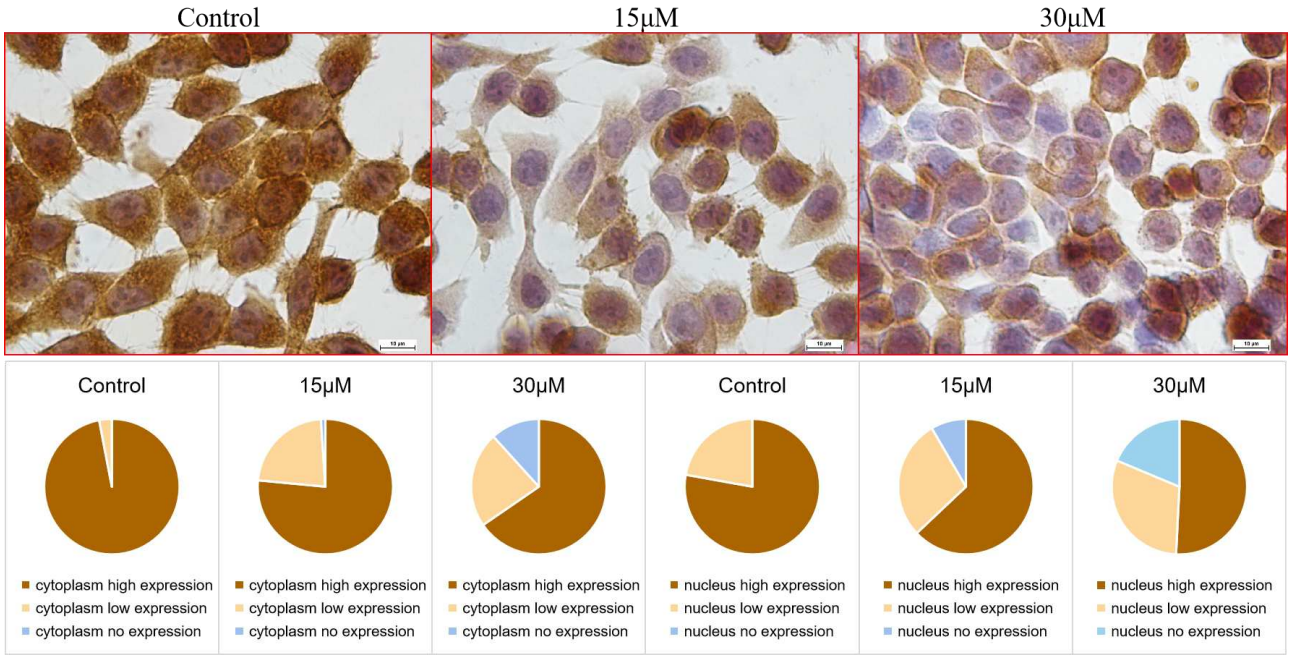


***Figure 11 Effect of solasonine on the expression of NFκB P65 protein in and out of the nucleus of HT-29 cells (1000× magnification):*** *After HT-29 cells were treated with solasonine for 48h, the immunocytochemistry assay was performed. And after observed under the microscope, cells with different levels of NFκB P65 expression were counted.*

As shown in Figure 12:

- The proportion of HCT116 cells with high expression of NFκB P65 protein in the cytoplasm was (1 ± 0)% in the control group, while that of 25μM group and 35μM group were (91.45 ± 3.45)% and (53.51 ± 15.48)% respectively, and there was a statistical difference between the control group and 35μM group (P< 0.05).

- The proportion of HCT116 cells with no expression of NFκB P65 protein in the cytoplasm was (0 ± 0)% in the control group, while that of 25μM group and 35μM group were (1.13 ± 1.70)% and (0.34 ± 0.76)% respectively, and there was no statistical difference between groups (P> 0.05).
- The proportion of HCT116 cells with high expression of NFκB P65 protein in the nucleus was (84.13 ± 7.20)% in the control group, while that of 25μM group and 35μM group were (67.83 ± 4.29)% and (35.43 ± 23.11)% respectively, and there was a statistical difference between groups (P< 0.05).
- The proportion of HCT116 cells with no expression of NFκB P65 protein in the nucleus was (0 ± 0)% in the control group, while that of 25μM group and 35μM group were (5.94 ± 1.45)% and (22.83 ± 15.52)% respectively, and there was a statistical difference between groups (P< 0.05).

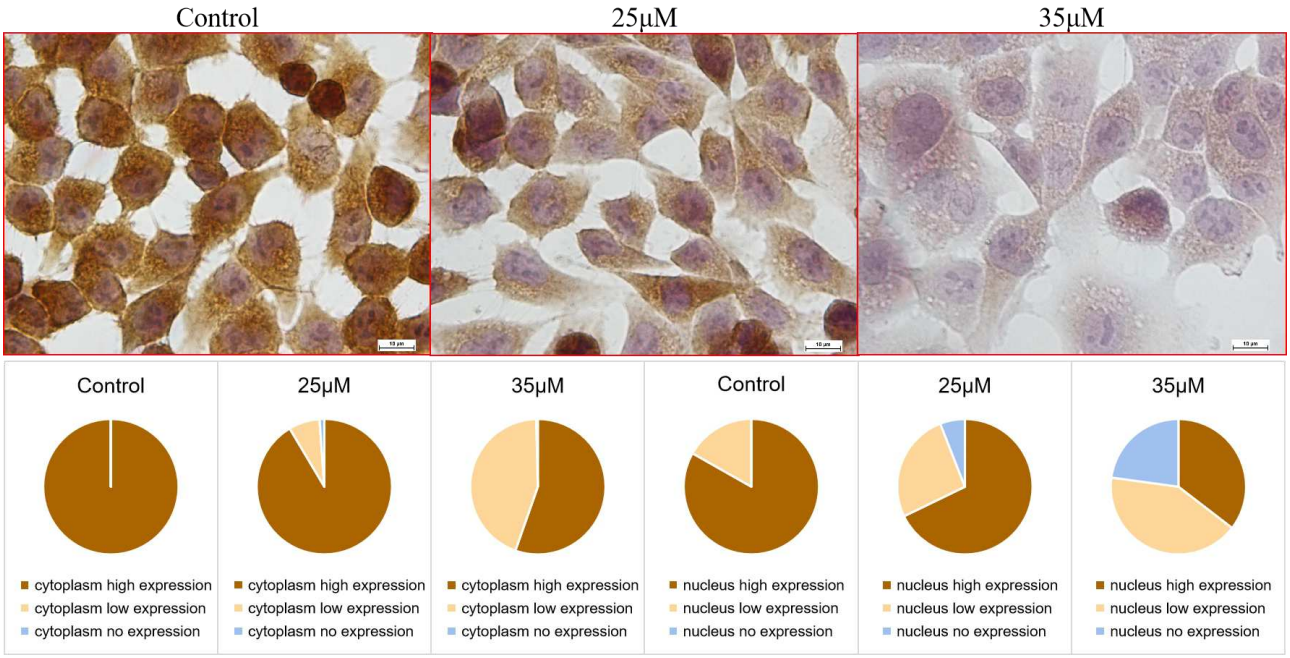


***Figure 12 Effect of solasonine on the expression of NFκB P65 protein in and out of the nucleus of HCT116 cells (1000× magnification):*** *After HCT116 cells were treated with solasonine for 48h, the immunocytochemistry assay was performed. And after observed under the microscope, cells with different levels of NFκB P65 expression were counted.*

As shown in Figure 13:

Only a few of the SW620 cells were fully adherent and spread out, while the rest were loosely adherent or appeared to be small spheres. The shape and characteristics of SW620 cells make them difficult to recognize and count in immunocytochemical images.


***Figure 13 SW620 cells in an immunocytochemical image (1000× magnification):*** *After SW620 cells were treated with solasonine for 48h, the immunocytochemistry assay was performed.*

As shown in Figure 14:

- After treated for 48h, the IOD/area (AOD) was 0.64 ± 0.09 in the control group of HT-29 cells while that in 15μM group and 30μM group were 0.46 ± 0.04 and 0.30 ± 0.02 respectively, and there was a statistical difference between groups ($P< 0.05$); it means the expression of p-NFκB P65 of HT-29 cells decreased with the increase of solasonine concentration.
- The AOD was 0.43 ± 0.02 in the control group of HCT116 cells while that in 25μM group and 35μM group were 0.20 ± 0.01 and 0.12 ± 0.03 respectively, and there was a statistical difference between groups ($P< 0.05$); it means the expression of p-NFκB P65 of HCT116 cells decreased with the increase of solasonine concentration.
- The shape and characteristics of SW620 cells make them difficult to recognize and measure in immunocytochemical images.

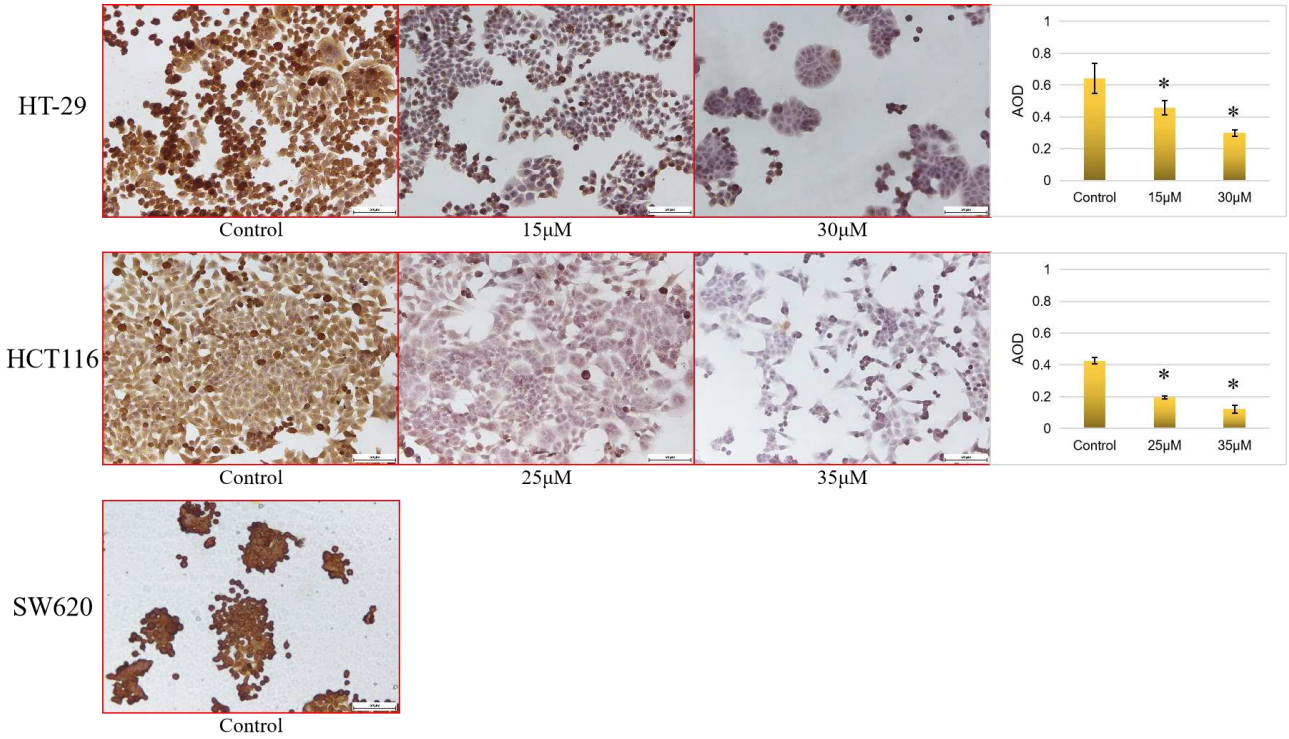


***Figure 14 Effect of solasonine on the expression of p-NFκB P65 protein of colon cancer cells (400× magnification):*** *After different cells were treated with solasonine for 48h, the immunocytochemistry assay was performed. And after observed under the microscope, the expression of p-NFκB P65 of each group was measured. But it was difficult to recognize the levels of p-NFκB P65 expression in SW620 cells (* p< 0.05 vs control group).*

## 5 Inhibition of solamargine on the proliferation of colon cancer cells

As shown in Figure 15:

- The inhibition rates of HT-29 cell proliferation were (14.13 ± 2.54)%, (25.47 ± 2.61)%, (57.58 ± 1.27)%, (80.57 ± 1.75)% and (90.30 ± 0.50)%, respectively, after treated with different concentrations of solamargine for 24h; the comparison between groups was statistically significant (P< 0.05), and the inhibition rate increased with the increase of solamargine concentration.
- The inhibition rates of SW620 cell proliferation were (4.31 ± 2.42)%, (17.48 ± 1.88)%, (69.94 ± 0.96)%, (84.41 ± 0.48)% and (86.09 ± 0.23)%, respectively, after treated with different concentrations of solamargine for 24h; the comparison between groups was statistically significant (P< 0.05), and the inhibition rate increased with the increase of solamargine concentration.

- The inhibition rates of HCT116 cell proliferation were (10.67 ± 3.5)%, (21.82 ± 2.95)%, (43.90 ± 2.65)%, (76.80 ± 0.21)% and (87.96 ± 0.15)%, respectively, after treated with different concentrations of solamargine for 24h; the comparison between groups was statistically significant (P< 0.05), and the inhibition rate increased with the increase of solamargine concentration.

According to the results above, we pick the concentration of solasonine as 3μM and 4μM for HT-29 cells, 2μM and 3μM for SW620 cells, 2μM and 4μM for HCT116 cells in next experiments, and the action time should be 24 hours.

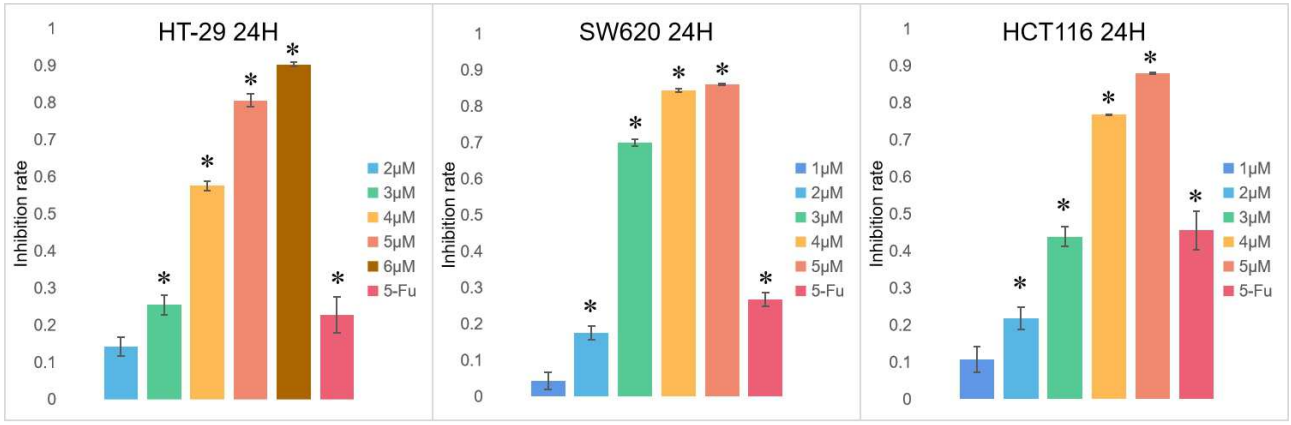


***Figure 15 Inhibition rates of solamargine on the proliferation of colon cancer cells:*** *After different cells were treated with solasmargine for 24h, the MTT assay was performed, and the inhibition rate of each group was calculated (* p< 0.05 vs control group).*

## 6 Solamargine induced apoptosis in colon cancer cells

As shown in Figure 16:

Microscopically, the nuclei of untreated cells (control group) were uniformly fluorescent and regular in shape; however, in the cells treated with solamargine, there were scattered nuclei wrinkled with enhancing fluorescence or several fluorescence fragments and scattered nuclei with dimming fluorescence.

The nucleus damage rate was measured:

- The nucleus damage rate of HT-29 cell control group was (0.85 ± 1.48)%, while that of 3μM group and 4μM group were (7.24 ± 2.80)% and (17.77 ± 4.42)% respectively; the

comparison between groups was statistically significant (P< 0.05), and the nucleus damage rate increased with the increase of solamargine concentration.

- The nucleus damage rate of SW620 cell control group was (1.12 ± 0.43)%, while that of 2μM group and 3μM group were (6.42 ± 1.03)% and (16.65 ± 0.95)% respectively; the comparison between groups was statistically significant (P< 0.05), and the nucleus damage rate increased with the increase of solamargine concentration.
- The nucleus damage rate of the HCT116 cell control group was (0.15 ± 0.26)%, while that of 2μM group and 4μM group were (7.97 ± 0.45)% and (19.55 ± 1.65)% respectively; the comparison between groups was statistically significant (P< 0.05), and the nucleus damage rate increased with the increase of solamargine concentration.

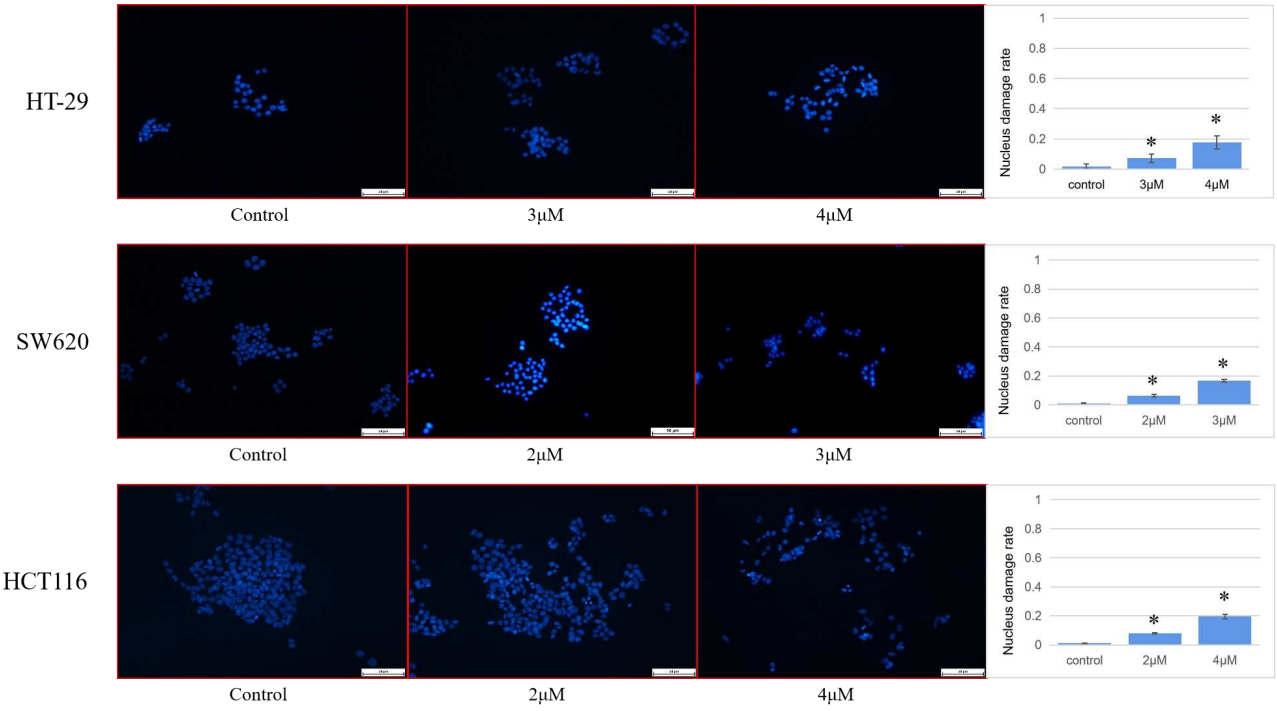


***Figure 16 The nucleus damage rate of colon cancer cells after solamargine treatment (400× magnification):*** *After different cells were treated with solamargine for 24h, the Hoechst* 33342 *staining assay was performed. And after observed under the fluorescence microscope, the nucleus damage rate of each group was measured (* p< 0.05 vs control group).*

As shown in Figure 17:

- In the HT-29 control group, pSIVA-IANBD positive areas accounted for (0.02 ± 0.002)% of the field of vision, while that in 3μM group and 4μM group accounted for (0.51 ± 0.04)% and (0.73 ± 0.02)% respectively; In the control group, PI-positive areas accounted for (0.03 ± 0.01)% of the field of vision, while that in 3μM group and 4μM group accounted for (0.18

± 0.03)% and (0.19 ± 0.06)% respectively; in pSIVA-IANBD staining, the comparison between groups was statistically significant (P< 0.05), and the positive areas increased with the increase of solamargine concentration; in PI staining, both 3μM group and 4μM group were significantly different from the control group (P< 0.05), but there was no difference between them (P> 0.05).

- In the SW620 control group, pSIVA-IANBD positive areas accounted for (0.04 ± 0.02)% of the field of vision, while that in 2μM group and 3μM group accounted for (0.16 ± 0.02)% and (0.92 ± 0.02)% respectively; In the control group, PI-positive areas accounted for (0.01 ± 0.002)% of the field of vision, while that in 2μM group and 3μM group accounted for (0.12 ± 0.04)% and (0.37 ± 0.07)% respectively; in both stainings, the comparison between groups was statistically significant (P< 0.05), and the positive areas increased with the increase of solamargine concentration.
- In the HCT116 control group, pSIVA-IANBD positive areas accounted for (0.02 ± 0.005)% of the field of vision, while that in 2μM group and 4μM group accounted for (0.40 ± 0.04)% and (0.77 ± 0.08)% respectively; In the control group, PI-positive areas accounted for (0.04 ± 0.02)% of the field of vision, while that in 2μM group and 4μM group accounted for (0.32 ± 0.05)% and (0.45 ± 0.07)% respectively; in both stainings, the comparison between groups was statistically significant (P< 0.05), and the positive areas increased with the increase of solamargine concentration.

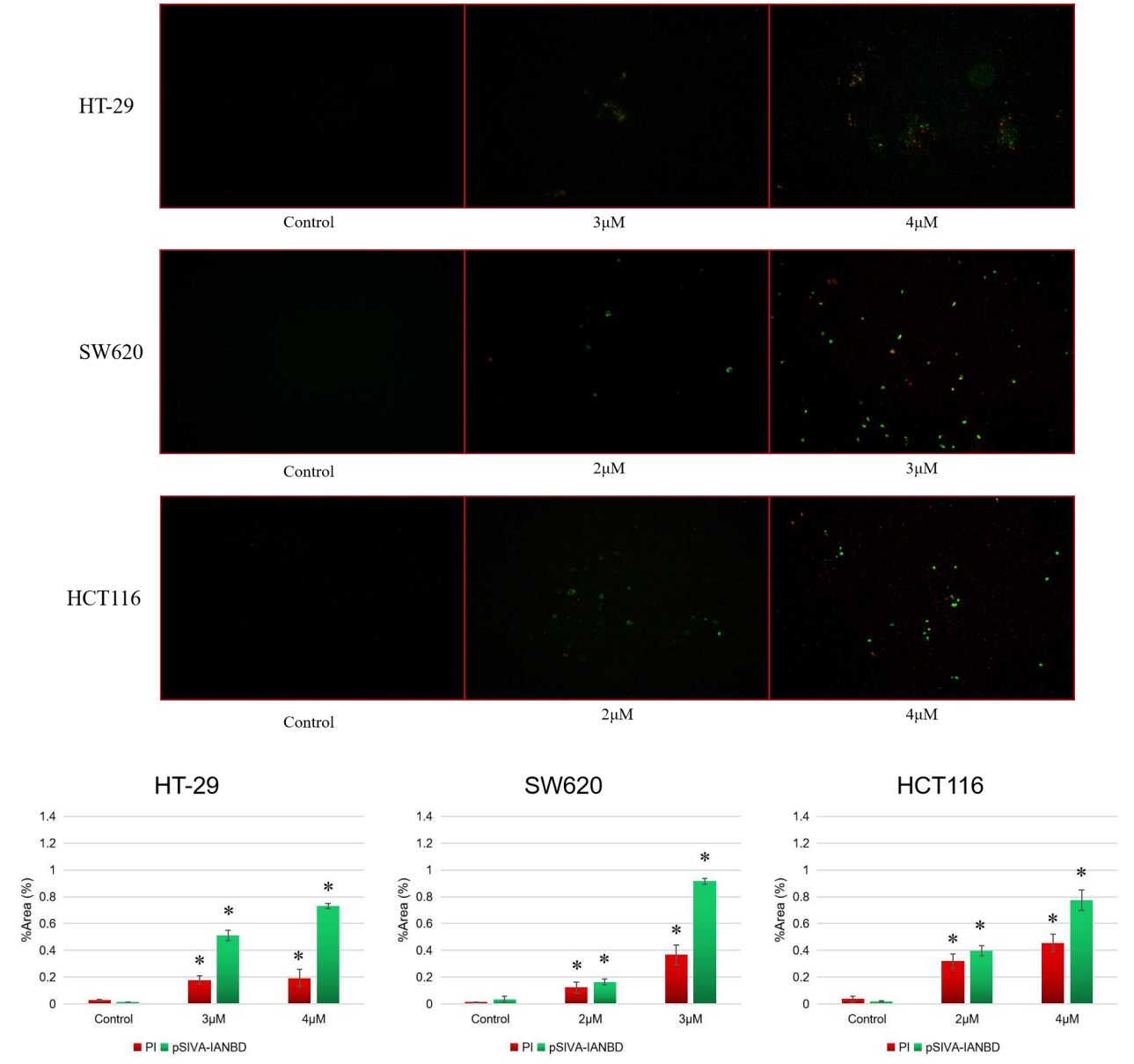


***Figure 17 The pSIVA-IANBD/ PI staining of colon cancer cells after solamargine 24h treatment (200× magnification):*** *After different cells were treated with solamargine for 24h, the pSIVA-IANBD/ PI staining assay was performed. And after observed under the fluorescence microscope, the positive area rate of each group was counted (* P< 0.05 vs control group).*

## 7 Expression of apoptosis-related proteins in colon cancer cells after solamargine treated

As shown in Figure 18:

After treated for 24 hours, the expression levels of IκB-α, pro-Caspase-3 and pro-Caspase-9 in HT-29 cells increased while p-IκB-α in the cytoplasm and NFκB P65 in nucleus decreased both in

3μM group and 4μM group; the expression of p-NFκB P65 decreased in 4μM group; the expression of Bcl-2 increased along with the increase of solamargine concentration.

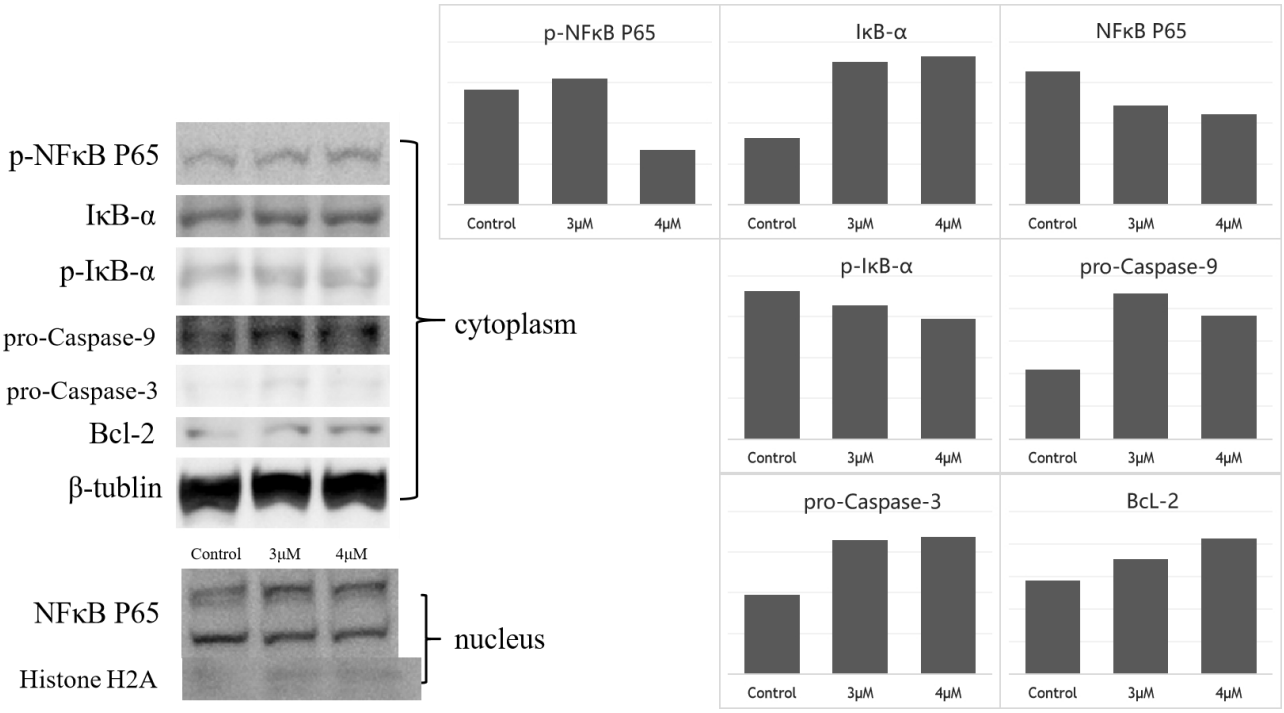


***Figure 18 Expression of apoptosis-related proteins in HT-29 cells after solamargine treatment:*** *After HT-29 cells were treated with solamargine for 24h, the Western blot assay was performed. And after reading by the gel imaging system, the gray value of each band was measured.*

As shown in Figure 19:

After treated for 24 hours, the expression levels of IκB-α in SW620 cells increased in 3μM group and that in the control group and 2μM group were similar, while p-IκB-α decreased in 3μM group; the expression of pro-Caspase-3 and p-NFκB P65 decreased as the concentration increased; the expression of NFκB P65 in nucleus decreased in 2μM group and 3μM group; the expression of Bcl-2 and pro-Caspase-9 were similar in the control group and 3μM group but increased in 2μM group.

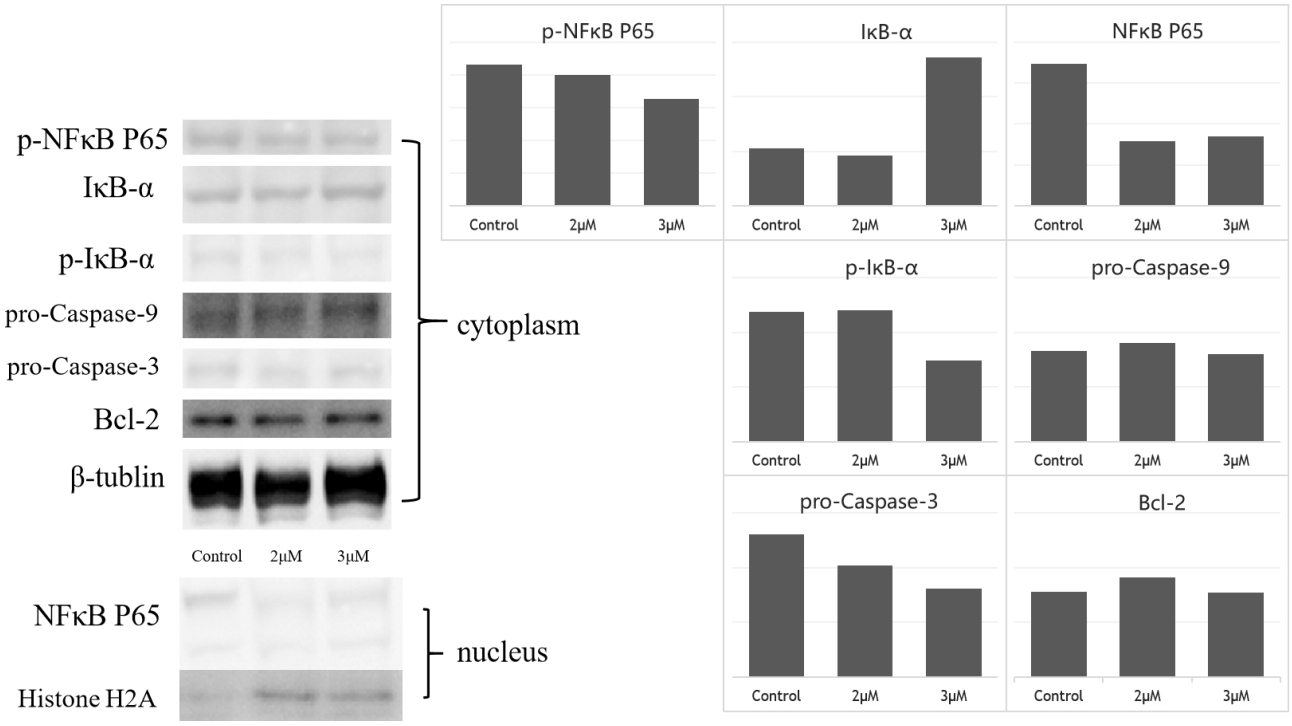


***Figure 19 Expression of apoptosis-related proteins in SW620 cells after solamargine treatment:*** *After SW620 cells were treated with solamargine for 24h, the Western blot assay was performed. And after reading by the gel imaging system, the gray value of each band was measured.*

As shown in Figure 20:

After treated for 24 hours, in HCT116 cells, the expression of p-NFκB P65 and pro-Caspase-9 in the cytoplasm and NFκB P65 in nucleus decreased as the concentration increased; the expression of IκB-α increased in 4μM group while p-IκB-α decreased; the expression of pro-Caspase-3 increased in 2μM group while it decreased in 3μM group; the expression of pro-Caspase-3 decreased in 2μM group while it increased in 3μM group.

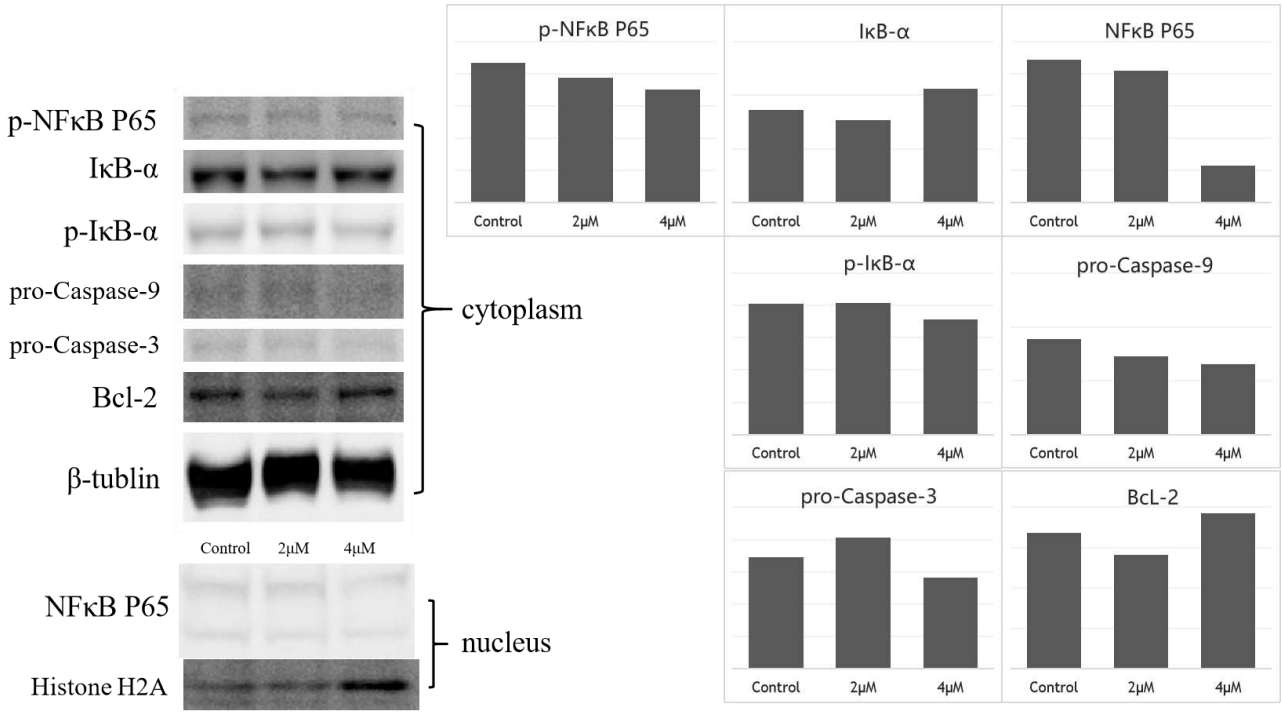


***Figure 20 Expression of apoptosis-related proteins in HCT116 cells after solamargine treatment:*** *After HCT116 cells were treated with solamargine for 24h, the Western blot assay was performed. And after reading by the gel imaging system, the gray value of each band was measured.*

The Western blot results showed that after solamargine treatment for 24h, the expression of pro-Caspase-3, pro-Caspase-9 and Bcl-2 showed different trends in diverse cell lines, and sometimes the change was not proportional to the drug concentration. However, similar to solasonine, these three cell lines all showed stable characteristics after treated: with high solamargine concentration, p-NFκB P65 and p-IκB-α as cytoplasmic proteins, NFκB P65 in nucleus decreased, and IκB-α in cytoplasm increased. According to this, immunocytochemistry was used to observe the effect of solamargine on the NFκB signaling pathway in colon cancer cells.

**8 The effect of solamargine on the NFκB signaling pathway**

As shown in Figure 21:

- The proportion of HT-29 cells with high expression of NFκB P65 protein in the cytoplasm was (100 ± 0)% in the control group, while that of 3μM group and 4μM group were (84.15 ± 4.48)% and (66.86 ± 7.80)% respectively, and there was a statistical difference between groups ($P< 0.05$).
- The amount of HT-29 cells with no expression of NFκB P65 protein in the cytoplasm was (0 ± 0)% in the control group, while that of the 3μM group and 4μM group were (0.90 ±

1.24)% and (15.80 ± 8.22)% respectively, and there was a statistical difference between the control group and 4μM group (P< 0.05) while there was no difference between the control group and 3μM group (P> 0.05).

- The percentage of HT-29 cells with high expression of NFκB P65 protein in the nucleus was (83.04 ± 1.46)% in the control group, while that of 3μM group and 4μM group were (66.86 ± 2.92)% and (57.53 ± 5.26)% respectively, and there was a statistical difference between groups (P< 0.05).
- The proportion of HT-29 cells with no expression of NFκB P65 protein in the nucleus was (0 ± 0)% in the control group, while that of 3μM group and 4μM group were (7.69 ± 3.49)% and (23.69 ± 7.18)% respectively, and there was a statistical difference between groups (P< 0.05).

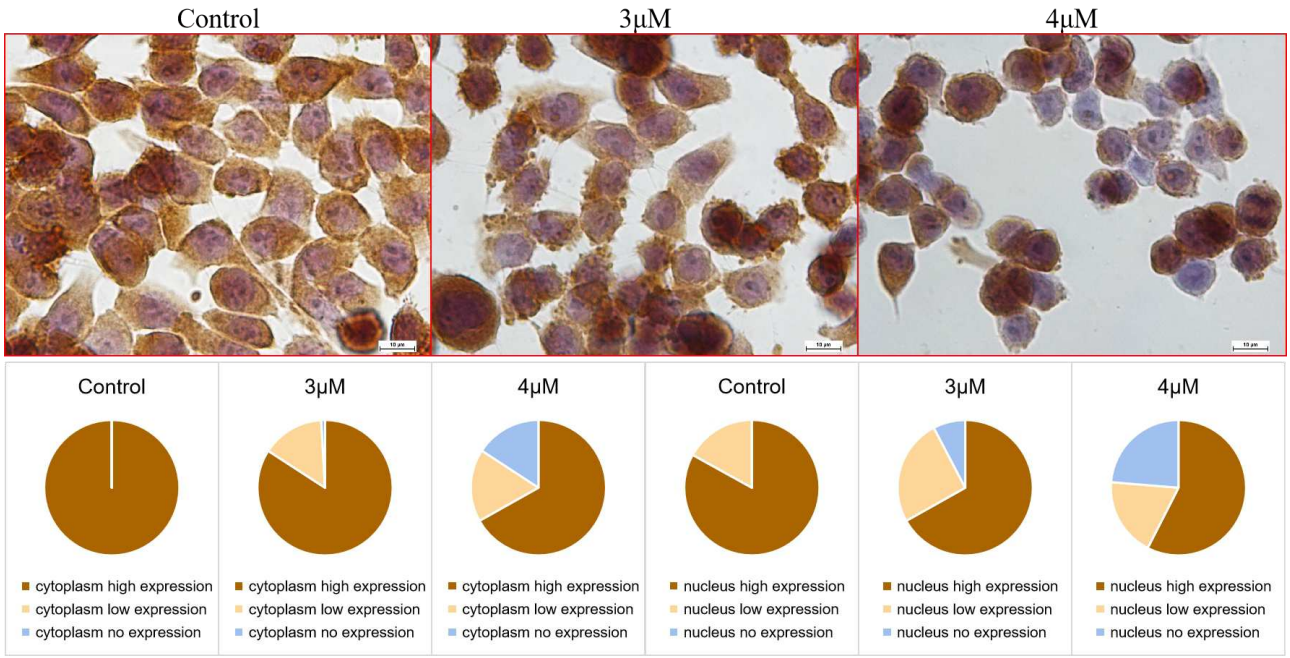


***Figure 21 Effect of solamargine on the expression of NFκB P65 protein in and out of the nucleus of HT-29 cells (1000× magnification):*** *After HT-29 cells were treated with solamargine for 24h, the immunocytochemistry assay was performed. And after observed under the microscope, cells with different levels of NFκB P65 expression were counted.*

As shown in Figure 22:

- The proportion of HCT116 cells with high expression of NFκB P65 protein in the cytoplasm was (1 ± 0)% in the control group, while that of 2μM group and 4μM group were (88.20 ± 2.78)% and (76.18 ± 6.36)% respectively, and there was a statistical difference between groups (P< 0.05).

- The percentage of HCT116 cells with no expression of NFκB P65 protein in the cytoplasm was (0 ± 0)% in the control group, while that of 2μM group and 4μM group were (0.28 ± 0.63)% and (0 ± 0)% respectively, and there was no statistical difference between groups (P> 0.05).
- The proportion of HCT116 cells with high expression of NFκB P65 protein in the nucleus was (87.48 ± 4.89)% in the control group, while that of 2μM group and 4μM group were (58.66 ± 9.54)% and (39.69 ± 9.75)% respectively, and there was a statistical difference between groups (P< 0.05).
- The percentage of HCT116 cells with no expression of NFκB P65 protein in the nucleus was (0 ± 0)% in the control group, while that of 2μM group and 4μM group were (3.65 ± 2.87)% and (9.01 ± 3.57)% respectively, and there was a statistical difference between groups (P< 0.05).

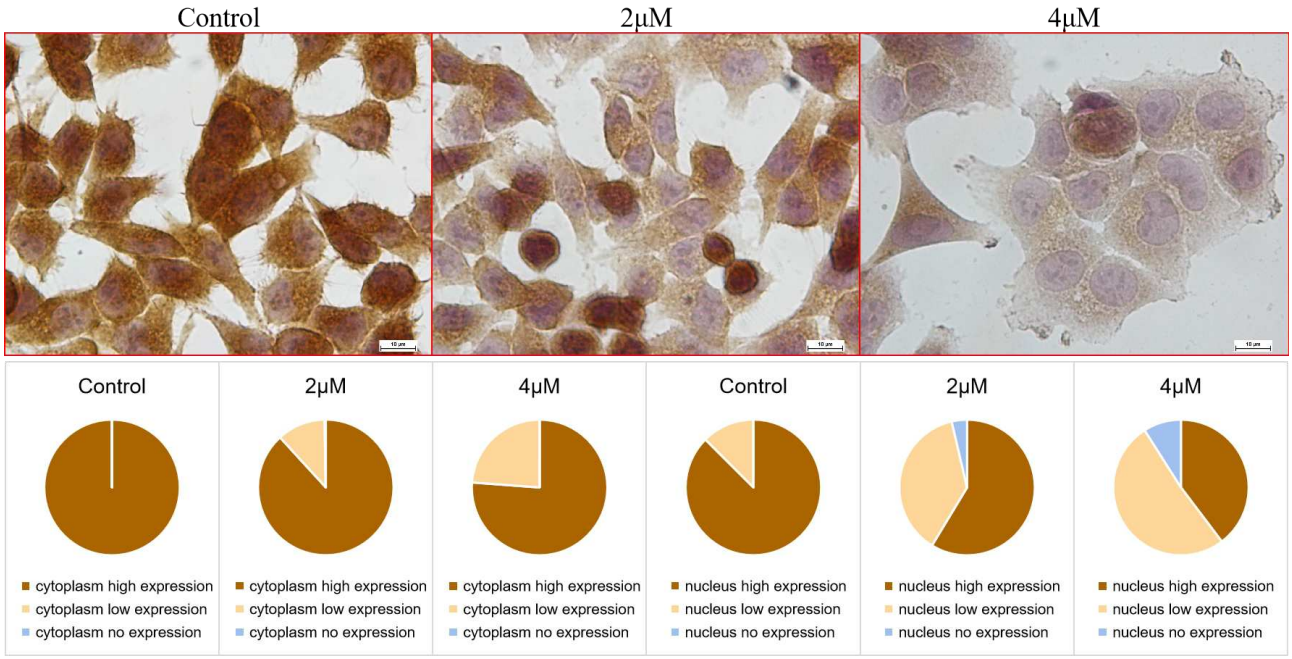


***Figure 22 Effect of solamargine on the expression of NFκB P65 protein in and out of the nucleus of HCT116 cells (1000× magnification):*** *After HCT116 cells were treated with solamargine for 24h, the immunocytochemistry assay was performed. And after observed under the microscope, cells with different levels of NFκB P65 expression were counted.*

As shown in Figure 23:

- After treated for 24h, the AOD was 0.59 ± 0.04 in the control group of HT-29 cells while that in 3μM group and 4μM group were 0.44 ± 0.01 and 0.39 ± 0.02 respectively, and there

was a statistical difference between groups (P< 0.05); it means the expression of p-NFκB P65 of HT-29 cells decreased with the increase of solamargine concentration.

- The AOD was 0.48 ± 0.07 in the control group of HCT116 cells while that in 2μM group and 4μM group were 0.20 ± 0.004 and 0.13 ± 0.01 respectively, and there was a statistical difference between groups (P< 0.05); it means the expression of p-NFκB P65 of HCT116 cells decreased with the increase of solamargine concentration.

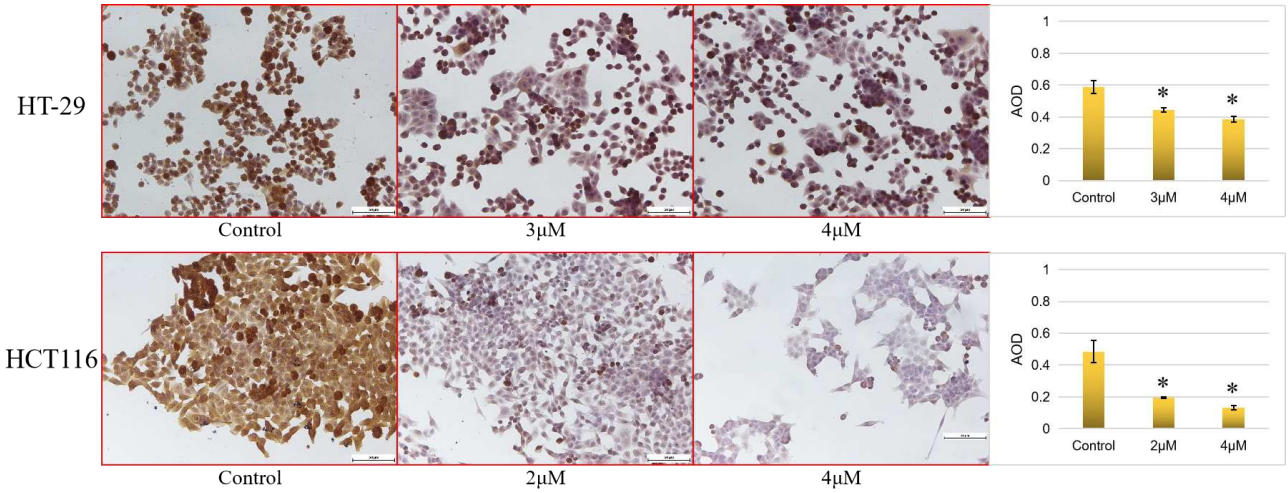


***Figure 23 Effect of solamargine on the expression of p-NFκB P65 protein of colon cancer cells (400× magnification):*** *After HT-29 and HCT116 cells were treated with solamargine for 24h, the immunocytochemistry assay was performed. And after observed under the microscope, the expression of p-NFκB P65 of each group was measured (* p< 0.05 vs control group).*

# Part III. Discussion

## 1 Discussion of results

The MTT results showed that the cell proliferation of the three cell lines was inhibited after 48h treatment with different concentrations of solasonine, and the inhibition rate was positively correlated with the concentration. Similarly, solamargine also showed a concentration dependent manner inhibition of the proliferation of colon cancer cells after 24h treatment. Both lower active concentration and shorter action time indicated that solamargine was more cytotoxic. In addition, solasonine appears to have a stronger inhibitory effect on HT-29 cells than on the other two less differentiated cell lines. On the contrary, solamargine had a stronger inhibitory effect on the two less differentiated cells than HT-29 cells.

The Hoechst 33342 staining showed that the number of damaged nuclei in the control groups of the three cell lines was very small, but increased after 48 hours of solasonine treatment, and the higher the concentration, the higher the nucleus damage rate. Solamargine also showed concentration dependent manner destruction of the cell nuclei in colon cancer. The results of pSIVA-IANBD/PI showed that there was almost no fluorescence signal of pSIVA-IANBD and PI in the control group of the three cell lines. After solasonine treated, pSIVA-IANBD staining of the three cell lines showed a concentration dependent manner increase, while PI staining was only increased in HT-29 cell line and SW620 cell line. And after solamargine treated, pSIVA-IANBD staining of the three cell lines also showed a concentration dependent manner increase, PI staining increased in each cell line but without difference between low concentration and high concentration in HT-29 cells. In addition, all groups treated with solasonine or solamargine always showed more green fluorescence than red fluorescence, indicating that some cells in each group were at an early stage of apoptosis. These results indicated that both solasonine and solamargine could induce apoptosis of the three cell lines, and the effect was positively correlated with the concentration.

Caspase-3 and Caspase-9 are active only when they break down from pro-Caspase-3,9 into cleaved Caspase-3,9. According to the trend of Western blot results, solasonine or solamargine did not decrease the pro-Caspase-3,9 of the three cell lines and even showed an increased trend in HT-29 and HCT116 cells. This makes it not reasonable to explain the mechanism of solasonine or solamargine induced apoptosis in colon cancer cells from the Caspase-9 /Caspase-3 pathway. Moreover, the irregular trend of anti-apoptotic protein Bcl-2 also suggested that the role of Bcl-2 in the process of solasonine or solamargine induced apoptosis of colon cancer cells was complex. However, as p-NFκB P65 and p-IκB-α in the cytoplasm, NFκB P65 in nucleus decreased, and IκB-

α in cytoplasm increased, this trend suggests the effect of solasonine or solamargine on the NFκB pathway: the reduction of phosphorylated IκB-α leads to the reduction of NFκB P65 liberated from IκB-α/NFκB P65 conformant and phosphorylated, thus reducing the number of NFκB P65 undergoing nucleus translocation. This suggests that solasonine or solamargine inhibits the NFκB pathway that inhibits apoptosis. To further verify this trend, immunocytochemistry was used to observe the effects of solasonine and solamargine on NFκB P65 phosphorylation and nuclear translocation in the three cell lines. However, the shape and characteristics of SW620 cells made them difficult to recognize and measure in immunocytochemical images. Thus, only the results of HT-29 and HCT116 cell lines could be discussed.

NFκB protein is abnormally high expressed in colon cancer cells, compared to normal colon epithelial cells, cancer cells exhibit aberrant constitutive NFκB activation which is involved in multiple signaling cascades related to carcinogenesis, including survival, invasion and migration [104]. The results of immunocytochemistry showed that the phosphorylation of NFκB P65 in HT-29 and HCT116 cells was decreased in a concentration-dependent manner after solasonine or solamargine treatment. The results confirmed the high expression of NFκB P65 in colon cancer cells and also demonstrated that the proportion of cells with high expression of NFκB P65 protein in the nucleus decreased while the proportion of cells with no expression of NFκB P65 protein in the nucleus increased with the rise of solasonine or solamargine concentration. It suggested that both solasonine and solamargine could inhibit the nucleus translocation of NFκB P65, thus inhibiting the role of the NFκB pathway.

From the perspective of inhibiting cell proliferation and inducing cell apoptosis, solamargine with greater cytotoxicity seems to have a better effect. However, in the case of unknown cytotoxicity to normal cells, or in vivo toxicity, the application prospect of the two drugs is difficult to determine.

## 2 Limitations and prospect

This research has some limitations: 1) There is a lack of detection of more apoptosis-related proteins. For example, Western blot results showed that apoptosis induced by solasonine and solamargine may be unrelated to the Caspase-9/Caspase-3 pathway, but it is not clear whether it is related to other proteins of the Caspase family, therefore achieved through other pathways of the Caspase family. 2) Detection of Bcl-2 protein was not convincing enough. The change of Bcl-2 protein alone could not explain the relationship between drug-induced apoptosis and Bcl-2 pathway.

This study attempted to detect the protein expression ratio of Bcl-2 to Bax but failed to achieve this goal.

In the future, we will improve the cell experiment part. Meanwhile, to improve and further study, in vivo preliminary experiment was carried out in China, as shown in Figure 24. And the results showed that both solasonine and solamargine have an excellent anti-tumor effect. Further in vivo experiments will be carried out to supplement the evidence. We hope to explore the deeper mechanism of action of solasonine and solamargine and explore their clinical application value through more detailed and objective studies.

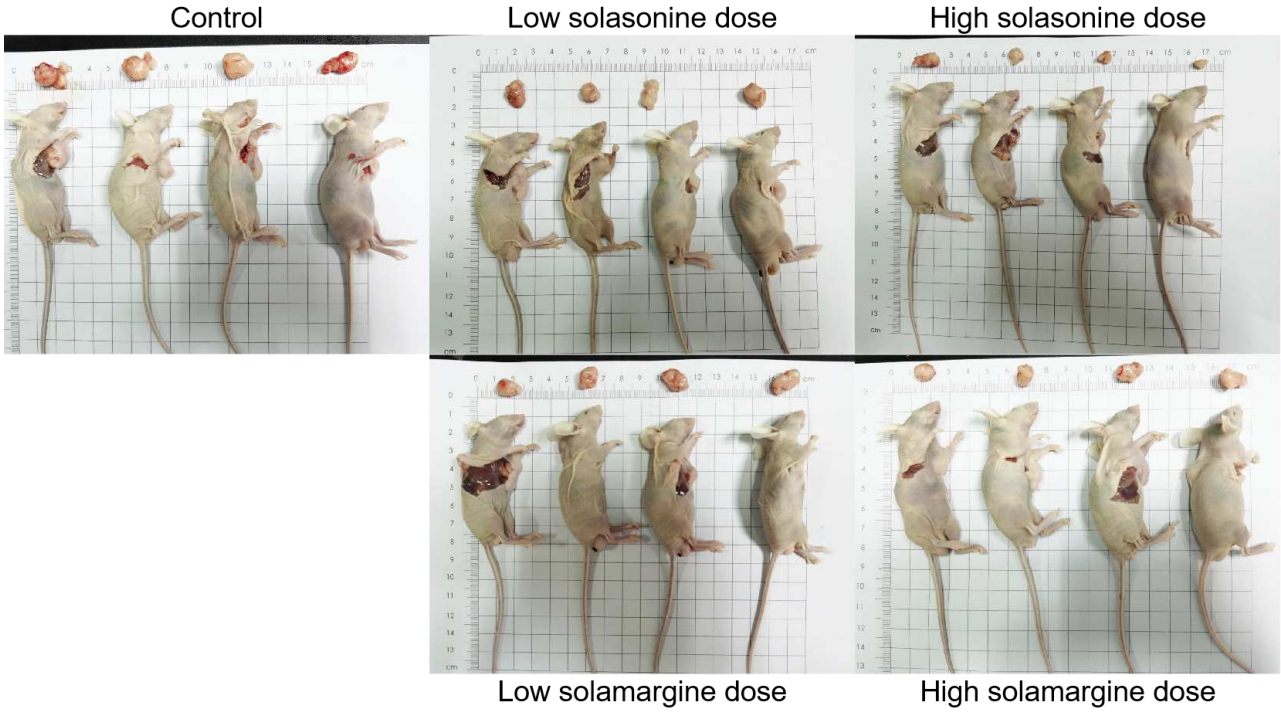


***Figure 24 In vivo study of solasonine and solamargine:*** *Twenty adult male nude mice were subcutaneously implanted with HCT116 cells and randomly divided into 5 groups (control group, low solasonine dose group, high solasonine dose group, low solamargine dose group, high solamargine dose group) with 4 mice in each group after tumor formation. Mice in each group were subcutaneously injected with normal saline, low dose solasonine, high dose solasonine, low-dose solamargine, and high dose solamargine, respectively. After one week, the mice were sacrificed by the neck-breaking method and the tumors were taken. The tumor volume of each mouse was measured and the average tumor volume was compared between groups. The results showed that compared with the control group, the tumor volumes were significantly smaller after solasonine or solamargine treatment. And for either solasonine treatment or solamargine treatment, the tumor volume of the high dose group was smaller than that of the low dose group. This indicates that both solasonine and solamargine have good anti-tumor effects in vivo.*

# Part IV. Conclusions

Based on theoretical and experimental studies, we can draw the following conclusions:

1) Solasonine can inhibit the proliferation of colon cancer cells with different degrees of differentiation, and the effect on HT-29 cells with relatively high degree of differentiation was more prominent.

2) Solasonine can induce apoptosis of colon cancer cells with different degrees of differentiation.

3) Solasonine can inhibit the NFκB pathway of colon cancer cells with different degrees of differentiation.

4) Solamargine can inhibit the proliferation of colon cancer cells with different degrees of differentiation and required drug concentration is smaller, action time is shorter.

5) Solamargine can induce apoptosis of colon cancer cells with different degrees of differentiation.

6) Solasonine can inhibit the NFκB pathway of colon cancer cells with different degrees of differentiation.

In conclusion, both solasonine and solamargine can inhibit the proliferation and induce apoptosis of colon cancer cells, and their effects are related to the NFκB pathway.

# References


[1]Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2020). Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed [02 01 2021].

[2]Dubey S, Siegfried JM, Traynor AM. Non-small-cell lung cancer and breast carcinoma: chemotherapy and beyond. Lancet Oncol. 2006 May;7(5):416-24. doi: 10.1016/S1470-2045(06)70693-3.

[3]Amjad MT, Kasi A. Cancer Chemotherapy. [Updated 2020 Nov 24]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2020 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK564367/

[4]Minniti G, Goldsmith C, Brada M. Radiotherapy. Handb Clin Neurol. 2012;104:215-28. doi: 10.1016/B978-0-444-52138-5.00016-5.

[5]Pastan I, Hassan R. Discovery of mesothelin and exploiting it as a target for immunotherapy. Cancer Res. 2014 Jun 1;74(11):2907-12. doi: 10.1158/0008-5472.CAN-14-0337.

[6]Sunshine J, Taube JM. PD-1/PD-L1 inhibitors. Curr Opin Pharmacol. 2015 Aug;23:32-8. doi: 10.1016/j.coph.2015.05.011.

[7]June CH, O'Connor RS, Kawalekar OU, Ghassemi S, Milone MC. CAR T cell immunotherapy for human cancer. Science. 2018 Mar 23;359(6382):1361-1365. doi: 10.1126/science.aar6711.

[8]Gegechkori N, Haines L, Lin JJ. Long-Term and Latent Side Effects of Specific Cancer Types. Med Clin North Am. 2017 Nov;101(6):1053-1073. doi: 10.1016/j.mcna.2017.06.003.

[9]Xiang Y, Guo Z, Zhu P, Chen J, Huang Y. Traditional Chinese medicine as a cancer treatment: Modern perspectives of ancient but advanced science. Cancer Med. 2019 May;8(5):1958-1975. doi: 10.1002/cam4.2108.

[10]Raju TN. The Nobel Chronicles. 1989: John Michael Bishop (b 1936) and Harold Eliot Varmus (b 1939). Lancet. 2000 Mar 25;355(9209):1106. doi: 10.1016/s0140-6736(05)72232-7.

[11]Schwartz D, Rotter V. p53-dependent cell cycle control: response to genotoxic stress. Semin Cancer Biol. 1998;8(5):325-36. doi: 10.1006/scbi.1998.0095.

[12]Owen KL, Brockwell NK, Parker BS. JAK-STAT Signaling: A Double-Edged Sword of Immune Regulation and Cancer Progression. Cancers (Basel). 2019 Dec 12;11(12):2002. doi: 10.3390/cancers11122002.

[13]Khan H, Reale M, Ullah H, Sureda A, Tejada S, Wang Y, Zhang ZJ, Xiao J. Anti-cancer effects of polyphenols via targeting p53 signaling pathway: updates and future directions. Biotechnol Adv. 2020 Jan-Feb;38:107385. doi: 10.1016/j.biotechadv.2019.04.007.

[14]Mitchell S, Vargas J, Hoffmann A. Signaling via the NFκB system. Wiley Interdiscip Rev Syst Biol Med. 2016 May;8(3):227-41. doi: 10.1002/wsbm.1331.

[15]Degirmenci U, Wang M, Hu J. Targeting Aberrant RAS/RAF/MEK/ERK Signaling for Cancer Therapy. Cells. 2020 Jan 13;9(1):198. doi: 10.3390/cells9010198.

[16]Ferrín G, Guerrero M, Amado V, Rodríguez-Perálvarez M, De la Mata M. Activation of mTOR Signaling Pathway in Hepatocellular Carcinoma. Int J Mol Sci. 2020 Feb 13;21(4):1266. doi: 10.3390/ijms21041266.

[17]Taciak B, Pruszynska I, Kiraga L, Bialasek M, Krol M. Wnt signaling pathway in development and cancer. J Physiol Pharmacol. 2018 Apr;69(2). doi: 10.26402/jpp.2018.2.07.

[18]Dituri F, Cossu C, Mancarella S, Giannelli G. The Interactivity between TGFβ and BMP Signaling in Organogenesis, Fibrosis, and Cancer. Cells. 2019 Sep 23;8(10):1130. doi: 10.3390/cells8101130.

[19]Lee S, Rauch J, Kolch W. Targeting MAPK Signaling in Cancer: Mechanisms of Drug Resistance and Sensitivity. Int J Mol Sci. 2020 Feb 7;21(3):1102. doi: 10.3390/ijms21031102.

[20]Macdonald RS, Wagner K. Influence of dietary phytochemicals and microbiota on colon cancer risk. J Agric Food Chem. 2012 Jul 11;60(27):6728-35. doi: 10.1021/jf204230r.

[21]Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer. Gastroenterology. 2010 Jun;138(6):2044-58. doi: 10.1053/j.gastro.2010.01.054.

[22]Keller DS, Windsor A, Cohen R, Chand M. Colorectal cancer in inflammatory bowel disease: review of the evidence. Tech Coloproctol. 2019 Jan;23(1):3-13. doi: 10.1007/s10151-019-1926-2.

[23]Van Cutsem E, Labianca R, Bodoky G, Barone C, Aranda E, Nordlinger B, Topham C, Tabernero J, André T, Sobrero AF, Mini E, Greil R, Di Costanzo F, Collette L, Cisar L, Zhang X, Khayat D, Bokemeyer C, Roth AD, Cunningham D. Randomized phase III trial comparing biweekly infusional fluorouracil/leucovorin alone or with irinotecan in the adjuvant treatment of stage III colon cancer: PETACC-3. J Clin Oncol. 2009 Jul 1;27(19):3117-25. doi: 10.1200/JCO.2008.21.6663.

[24]Ychou M, Raoul JL, Douillard JY, Gourgou-Bourgade S, Bugat R, Mineur L, Viret F, Becouarn Y, Bouché O, Gamelin E, Ducreux M, Conroy T, Seitz JF, Bedenne L, Kramar A. A phase III randomised trial of LV5FU2 + irinotecan versus LV5FU2 alone in adjuvant high-risk colon cancer (FNCLCC Accord02/FFCD9802). Ann Oncol. 2009 Apr;20(4):674-80. doi: 10.1093/annonc/mdn680.

[25]André T, Vernerey D, Im SA, Bodoky G, Buzzoni R, Reingold S, Rivera F, McKendrick J, Scheithauer W, Ravit G, Fountzilas G, Yong WP, Isaacs R, Österlund P, Liang JT, Creemers GJ, Rakez M, Van Cutsem E, Cunningham D, Tabernero J, de Gramont A. Bevacizumab as adjuvant treatment of colon cancer: updated results from the S-AVANT phase III study by the GERCOR Group. Ann Oncol. 2020 Feb;31(2):246-256. doi: 10.1016/j.annonc.2019.12.006.

[26]Stintzing S, Wirapati P, Lenz HJ, Neureiter D, Fischer von Weikersthal L, Decker T, Kiani A, Kaiser F, Al-Batran S, Heintges T, Lerchenmüller C, Kahl C, Seipelt G, Kullmann F, Moehler M, Scheithauer W, Held S, Modest DP, Jung A, Kirchner T, Aderka D, Tejpar S, Heinemann V.

Consensus molecular subgroups (CMS) of colorectal cancer (CRC) and first-line efficacy of FOLFIRI plus cetuximab or bevacizumab in the FIRE3 (AIO KRK-0306) trial. Ann Oncol. 2019 Nov 1;30(11):1796-1803. doi: 10.1093/annonc/mdz387.

[27]Martenson JA Jr, Willett CG, Sargent DJ, Mailliard JA, Donohue JH, Gunderson LL, Thomas CR Jr, Fisher B, Benson AB 3rd, Myerson R, Goldberg RM. Phase III study of adjuvant chemotherapy and radiation therapy compared with chemotherapy alone in the surgical adjuvant treatment of colon cancer: results of intergroup protocol 0130. J Clin Oncol. 2004 Aug 15;22(16):3277-83. doi: 10.1200/JCO.2004.01.029.

[28]Lee JJ, Chu E. The Adjuvant Treatment of Stage III Colon Cancer: Might Less Be More? Oncology (Williston Park). 2018 Sep 15;32(9):437-42, 444.

[29]Newman NB, Moss RA, Maloney-Patel N, Donohue K, Brown TV, Nissenblatt MJ, Lu SE, Jabbour SK. Bone marrow tolerance during postoperative chemotherapy in colorectal carcinomas. J Gastrointest Oncol. 2017 Jun;8(3):547-555. doi: 10.21037/jgo.2017.01.19.

[30]Verhaar S, Vissers PA, Maas H, van de Poll-Franse LV, van Erning FN, Mols F. Treatment-related differences in health related quality of life and disease specific symptoms among colon cancer survivors: results from the population-based PROFILES registry. Eur J Cancer. 2015 Jul;51(10):1263-73. doi: 10.1016/j.ejca.2015.04.004.

[31]Wang CC, Zhu R, Tan JY. Nurses and Holistic Modalities: The History of Chinese Medicine and Acupuncture. Holist Nurs Pract. 2019 Mar/Apr;33(2):90-94. doi: 10.1097/HNP.0000000000000312.

[32]Li Z, Xu C. The fundamental theory of traditional Chinese medicine and the consideration in its research strategy. Front Med. 2011 Jun;5(2):208-11. doi: 10.1007/s11684-011-0126-x.

[33]Spill F, Reynolds DS, Kamm RD, Zaman MH. Impact of the physical microenvironment on tumor progression and metastasis. Curr Opin Biotechnol. 2016 Aug;40:41-48. doi: 10.1016/j.copbio.2016.02.007.

[34]Ji Q, Luo YQ, Wang WH, Liu X, Li Q, Su SB. Research advances in traditional Chinese medicine syndromes in cancer patients. J Integr Med. 2016 Jan;14(1):12-21. doi: 10.1016/S2095-4964(16)60237-6.

[35]Shu P, Tang H, Zhou B, Wang R, Xu Y, Shao J, Qi M, Xia Y, Huang W, Liu S. Effect of Yiqi Huayu Jiedu decoction on stages II and III gastric cancer: A multicenter, prospective, cohort study. Medicine (Baltimore). 2019 Nov;98(47):e17875. doi: 10.1097/MD.0000000000017875.

[36]Zhou L, Zhu XZ, Xia JH et al. Clinical Study on Yiqi Yangyin Recipe in Treating the Adverse Effects after the Chemotherapy in the Tumor Patients. Clinical Journal of Traditional Chinese Medicine 07, 1072-1075, 2017. doi: 10.16448/j.cjtcm.2017.0359. (in Chinese)

[37]Qiao ZA. Effects of Yiqi Yangyin Prescription combined with radiotherapy on the tumor load and antitumor immune response in patients with advanced lung cancer. Journal of Hainan Medical University 01, 75-78, 2018. doi: 10.13210/j.cnki.jhmu.20171227.002. (in Chinese)

[38]Duan M, Jia CF, Duan X. Effects of Yiqi Gu Ben Decoction combined with DC chemotherapy on serum tumor markers, inflammatory factors and immune function in patients with locally advanced non-small cell lung cancer. Journal of Hainan Medical University 01, 79-82, 2018. doi: 10.13210/j.cnki.jhmu.20171127.007. (in Chinese)

[39]Song M, Xi SS, Liu JP et al. Effects of Shenqi Fuzheng Injection on Immune Function and Toxic Reactions in Chemotherapy Patients after Rectum Carcinoma Resection. Guiding Journal of Traditional Chinese Medicine and Pharmacy 02, 46-48, 2015. doi: 10.13862/j.cnki.cn43-1446/r.2015.02.017. (in Chinese)

[40]Ma JJ, Liu HP. Efficacy of Aidi Injection () on overexpression of P-glycoprotein induced by vinorelbine and cisplatin regimen in patients with non-small cell lung cancer. Chin J Integr Med. 2017 Jul;23(7):504-509. doi: 10.1007/s11655-016-2511-4.

[41]Meng H, Sun X, Nian JY et al. Sodium Cantharidate Combined with Chemotherapy in Advanced Gastric Cancer: A Meta-analysis. World Chinese Medicine 10, 2496-2500, 2017. doi: 10.3969/j.issn.1673-7202.2017.10.055. (in Chinese)

[42]Liu F, Hu WS, Ye XW et al. Clinical efficacy of Xiaoaiping injection as retention enema in the treatment of colorectal cancer. Chinese Journal of General Practice 11, 1831-1835, 2014. doi: 10.16766/j.cnki.issn.1674-4152.2014.11.009. (in Chinese)

[43]Lin Y, Ma L, Bulibu J, Kebinu M. Application of Traditional Chinese Medicine in the Adjuvant Treatment of Colon Cancer. China Pharmacist 21, 1637-1640, 2018. (in Chinese)

[44]Zhang ZL, Hong L, Fang MZ. Research Progress of Chinese Medicine Combined with Chemotherapy in Treatment of Colorectal Cancer. Journal of Liaoning University of TCM 21, 221-224, 2019. doi: 10.13194/j.issn.1673-842x.2019.09.058. (in Chinese)

[45]Zhang T, He WT, Yang YF. Literature Study on Chinese Medicine Syndrome of IV-stage Colorectal Cancer and Related Prescription Laws. Journal of Traditional Chinese Medicine 58, 966-970, 2017. doi: 10.13288/j.11-2166/r.2017.11.016. (in Chinese)

[46] Niu H, Liu J. Advances in TCM treatment for metastasis of tumors. J Tradit Chin Med. 2003 Jun;23(2):151-7.

[47]Peng TS, Xie B, Nie T, Hu XD, Yang ZL, Li SJ, Wang XY, Luo M, He YH. Analysis of clinical effects about Tianma granules inhibition of tumor recurrence in colorectal cancer. Pharmacology and Clinics of Chinese Materia Medica 32, 191-193, 2016. doi: 10.13412/j.cnki.zyyl.2016.03.056. (in Chinese)

[48]Xi JL, Shi L, Zhang CX, Li ZY, Peng B. Effect of Chinese Medicine Invigorating Spleen Combined with FOLFOX Chemotherapy on Cancer-Related Fatigue, Th1 /Th2 Immune Response Balance and Peripheral Neuropathy in Patients with Colon Cancer after Operation. Chinese Archives of Traditional Chinese Medicine 37, 1503-1505, 2019. doi: 10.13193/j.issn.1673-7717.2019.06.057. (in Chinese)

[49]Wang RR, Wang QM, Jiang YL, Yang P. Advanced metastatic colorectal cancer treated with a combination of Jianpi Xiaoai Formula and chemotherapy. China Journal of Traditional Chinese Medicine and Pharmacy 31, 1732-1736, 2016. (in Chinese)

[50]Yu L, Zhou Y, Yang Y, Lu F, Fan Y. Efficacy and Safety of Compound Kushen Injection on Patients with Advanced Colon Cancer: A Meta-Analysis of Randomized Controlled Trials. Evid Based Complement Alternat Med. 2017;2017:7102514. doi: 10.1155/2017/7102514.

[51]Ge Q, Yang ZT, Wang M, Meng JY. In Vitro Study on Reversal of Multidrug Resistance of Colorectal Cancer Cells by Xihuang Pills. Chinese Archives of Traditional Chinese Medicine 36, 176-180, 2018. doi: 10.13193/j.issn.1673-7717.2018.01.043. (in Chinese)

[52]Langmead L, Rampton DS. Review article: complementary and alternative therapies for inflammatory bowel disease. Aliment Pharmacol Ther. 2006 Feb 1;23(3):341-9. doi: 10.1111/j.1365-2036.2006.02761.x.

[53]Wu AP. Effect of Qingchang Xiaoai granule enema combined with chemotherapy on the short-term efficacy and quality of life of patients with advanced colorectal cancer. Chinese Journal of Modern Drug Application 14, 11-13, 2020. doi: 10.14164/j.cnki.cn11-5581/r.2020.22.005. (in Chinese)

[54]Gao YN, Yu XY. Observation on the curative effect of Traditional Chinese Medicine enema in the treatment of intestinal paralysis after laparoscopic colon cancer operation. Electronic Journal of Clinical Medical Literature 6, 159, 2019. doi: 10.16281/j.cnki.jocml.2019.30.140. (in Chinese)

[55]Lu M, Wang C, Wang J. Tanshinone I induces human colorectal cancer cell apoptosis: The potential roles of Aurora A-p53 and survivin-mediated signaling pathways. Int J Oncol. 2016 Aug;49(2):603-10. doi: 10.3892/ijo.2016.3565.

[56]Tao Y, Zhan S, Wang Y, Zhou G, Liang H, Chen X, Shen H. Baicalin, the major component of traditional Chinese medicine Scutellaria baicalensis induces colon cancer cell apoptosis through inhibition of oncomiRNAs. Sci Rep. 2018 Sep 27;8(1):14477. doi: 10.1038/s41598-018-32734-2.

[57]Tang YC, Zhang Y, Zhou J, Zhi Q, Wu MY, Gong FR, Shen M, Liu L, Tao M, Shen B, Gu DM, Yu J, Xu MD, Gao Y, Li W. Ginsenoside Rg3 targets cancer stem cells and tumor angiogenesis to inhibit colorectal cancer progression in vivo. Int J Oncol. 2018 Jan;52(1):127-138. doi: 10.3892/ijo.2017.4183.

[58]Yuan Z, Shi X, Qiu Y, Jia T, Yuan X, Zou Y, Liu C, Yu H, Yuan Y, He X, Xu K, Yin P. Reversal of P-gp-mediated multidrug resistance in colon cancer by cinobufagin. Oncol Rep. 2017 Mar;37(3):1815-1825. doi: 10.3892/or.2017.5410.

[59]Pacioni S, D'Alessandris QG, Giannetti S, Morgante L, De Pascalis I, Coccè V, Bonomi A, Pascucci L, Alessandri G, Pessina A, Falchetti ML, Pallini R. Mesenchymal stromal cells loaded with paclitaxel induce cytotoxic damage in glioblastoma brain xenografts. Stem Cell Res Ther. 2015 Oct 6;6:194. doi: 10.1186/s13287-015-0185-z.

[60]Zhao X, Zhang Q, Chen L. Triptolide induces the cell apoptosis of osteosarcoma cells through the TRAIL pathway. Oncol Rep. 2016 Sep;36(3):1499-505. doi: 10.3892/or.2016.4957.

[61]Liu ZH, Wang G, Shen JF. Mechanism of Saikosaponin-d Inhibiting Cervical Cell Hela. Chinese Archives of Traditional Chinese Medicine 12, 2931-2934, 2016. doi: 10.13193/j.issn.1673-7717.2016.12.031. (in Chinese)

[62]Liu J, Wen X, Liu B, Zhang Q, Zhang J, Miao H, Zhu R. Diosmetin inhibits the metastasis of hepatocellular carcinoma cells by downregulating the expression levels of MMP-2 and MMP-9. Mol Med Rep. 2016 Mar;13(3):2401-8. doi: 10.3892/mmr.2016.4872. Epub 2016 Feb 5.

[63]Wan XY, Zhai XF, Jiang YP, Han T, Zhang QY, Xin HL. Antimetastatic effects of norcantharidin on hepatocellular carcinoma cells by up-regulating FAM46C expression. Am J Transl Res. 2017 Jan 15;9(1):155-166.

[64]Huang T, Xiao Y, Yi L, Li L, Wang M, Tian C, Ma H, He K, Wang Y, Han B, Ye X, Li X. Coptisine from Rhizoma Coptidis Suppresses HCT-116 Cells-related Tumor Growth in vitro and in vivo. Sci Rep. 2017 Feb 6;7:38524. doi: 10.1038/srep38524.

[65]Fu Z, Chen X, Guan S, Yan Y, Lin H, Hua ZC. Curcumin inhibits angiogenesis and improves defective hematopoiesis induced by tumor-derived VEGF in tumor model through modulating VEGF-VEGFR2 signaling pathway. Oncotarget. 2015 Aug 14;6(23):19469-82. doi: 10.18632/oncotarget.3625.

[66]Xiao W, Wu K, Yin M, Han S, Ding Y, Qiao A, Lu G, Deng B, Bo P, Gong W. Wogonin Inhibits Tumor-derived Regulatory Molecules by Suppressing STAT3 Signaling to Promote Tumor Immunity. J Immunother. 2015 Jun;38(5):167-84. doi: 10.1097/CJI.0000000000000080.

[67]Sui H, Zhou LH, Zhang YL, Huang JP, Liu X, Ji Q, Fu XL, Wen HT, Chen ZS, Deng WL, Zhu HR, Li Q. Evodiamine Suppresses ABCG2 Mediated Drug Resistance by Inhibiting p50/p65 NF-κB Pathway in Colorectal Cancer. J Cell Biochem. 2016 Jun;117(6):1471-81. doi: 10.1002/jcb.25451. Epub 2016 Feb 29.

[68]Buhrmann C, Shayan P, Kraehe P, Popper B, Goel A, Shakibaei M. Resveratrol induces chemosensitization to 5-fluorouracil through up-regulation of intercellular junctions, Epithelial-to-mesenchymal transition and apoptosis in colorectal cancer. Biochem Pharmacol. 2015 Nov 1;98(1):51-68. doi: 10.1016/j.bcp.2015.08.105.

[69]Zhang B, Chen N, Chen H, Wang Z, Zheng Q. The critical role of redox homeostasis in shikonin-induced HL-60 cell differentiation via unique modulation of the Nrf2/ARE pathway. Oxid Med Cell Longev. 2012;2012:781516. doi: 10.1155/2012/781516.

[70]Chen H, Zhang B, Yao Y, Chen N, Chen X, Tian H, Wang Z, Zheng Q. NADPH oxidase-derived reactive oxygen species are involved in the HL-60 cell monocytic differentiation induced by isoliquiritigenin. Molecules. 2012 Nov 12;17(11):13424-38. doi: 10.3390/molecules171113424.

[71]Fujiwara Y, Shiraishi D, Yoshitomi M et al. Soyasapogenols contained in soybeans suppress tumour progression by regulating macrophage differentiation into the protumoural phenotype. J Funct Foods 19, 594-605, 2015. https://doi.org/10.1016/j.jff.2015.09.055.

[72]Chang WT, Lai TH, Chyan YJ, Yin SY, Chen YH, Wei WC, Yang NS. Specific medicinal plant polysaccharides effectively enhance the potency of a DC-based vaccine against mouse mammary tumor metastasis. PLoS One. 2015 Mar 31;10(3):e0122374. doi: 10.1371/journal.pone.0122374.

[73]Li J, Liu XB. Advances in medicine of toxicity and synergistic effects in cancer treatment. Journal of Pharmaceutical Research 04, 229-231, 2015. doi: 10.13506/j.cnki.jpr.2015.04.014. (in Chinese)

[74]Zhou CH, Chen G, Zeng L et al. Clinical Observation of Shengbai Oral Solution on Prevention of Neutropenia Induced by Chemotherapy for Lung Cancers. Chinese Journal of Surgery of Integrated Traditional and Western Medicine 02, 131-134, 2018. doi: 10.3969/j.issn.1007-6948.2018.02.001. (in Chinese)

[75]Wei GH, Xu CM, Yang Y. Effect of Longkui Tingli Decoction Combined with Cisplatin Chemotherapy on Lung Cancer Patients with Massive Pleural Effusion and Its Influence on Immune Indexes. Medical Recapitulate 26, 1244-1248, 2020. (in Chinese)

[76]Huang DB, Guan J. Clinical study of Longkui mixture on quality of life and immune function of patients with advanced liver cancer. Lishizhen Medicine and Materia Medica Research 24, 1676-1678, 2013. doi: 10.3969/j.issn.1008-0805.2013.07.058. (in Chinese)

[77]Zhong MW, Ye HQ. Clinical research of Qifu Longkui decoction combined with chemotherapy in the treatment of metastatic colorectal cancer. Chinese Journal of Modern Drug Application 10, 1-3, 2016. doi: 10.14164/j.cnki.cn11-5581/r.2016.09.001. (in Chinese)

[78]Wang SL. Effect of Qifu Longkui decoction combined with FOLFOX4 regimen on serum tumor markers, bone marrow suppression and immune function in patients with metastatic colorectal cancer. Journal of Logistics University of PAP (Medical Sciences) 27, 505-508, 2018. doi:10.16548/j.2095-3720.2018.06.011. (in Chinese)

[79]Tong JB, Zhang XX, Wang XH, Zeng SJ, Wang DY, Zhang ZQ, Hu J, Yang C, Li ZG. Qiyusanlong decoction suppresses lung cancer in mice via Wnt/β-catenin pathway. Mol Med Rep. 2018 Apr;17(4):5320-5327. doi: 10.3892/mmr.2018.8478.

[80]Zhao XZ. Apoptosis of Cervical Cancer HeLa Cells Induced by Solanum solanum and Its Mechanism. World Latest Medicine Information (Electronic Version) 17, 102-104, 2017. doi: 10.3969/j.issn.1671-3141.2017.42.060. (in Chinese)

[81]Shokrzadeh M, Azadbakht M, Ahangar N, Hashemi A, Saeedi Saravi SS. Cytotoxicity of hydro-alcoholic extracts of Cucurbitapepo and Solanum nigrum on HepG2 and CT26 cancer cell lines. Pharmacogn Mag. 2010 Jul;6(23):176-9. doi: 10.4103/0973-1296.66931.

[82]Wang W, Lu DP. An in vitro study of cytotoxic and antineoplastic effect of Solanum nigrum Lextract on U266. Journal of Peking University (Health Sciences) 37, 240-244, 2005. (in Chinese)

[83]Nawab A, Thakur VS, Yunus M, Ali Mahdi A, Gupta S. Selective cell cycle arrest and induction of apoptosis in human prostate cancer cells by a polyphenol-rich extract of Solanum nigrum. Int J Mol Med. 2012 Feb;29(2):277-84. doi: 10.3892/ijmm.2011.835.

[84]Wang CK, Lin YF, Tai CJ, Wang CW, Chang YJ, Choong CY, Lin CS, Tai CJ, Chang CC. Integrated Treatment of Aqueous Extract of Solanum nigrum-Potentiated Cisplatin- and Doxorubicin-Induced Cytotoxicity in Human Hepatocellular Carcinoma Cells. Evid Based Complement Alternat Med. 2015;2015:675270. doi: 10.1155/2015/675270.

[85]Yu C, Liu SL, Zou X, Wu J. Clinical Research on Guanchang Fang's Inhibit Function on the Growth of Advanced intestinal Cancer and the Expression of Serum VEGF and miRNA31. Journal of Nanjing University of Traditional Chinese Medicine 30, 33-36, 2014. doi: 10.14148/j.issn.1672-0482.2014.01.014. (in Chinese)

[86]Yu C, Xu MJ, Ju WZ. Liu SL. Active Ingredient of Enema in Treatment of Colorectal Cancer. Journal of Liaoning University of Traditional Chinese Medicine 16, 50-54, 2014. doi: 10.13194/j.issn.1673-842x.2014.05.017. (in Chinese)

[87]Friedman M. Chemistry and anticarcinogenic mechanisms of glycoalkaloids produced by eggplants, potatoes, and tomatoes. J Agric Food Chem. 2015 Apr 8;63(13):3323-37. doi: 10.1021/acs.jafc.5b00818.

[88]Wang X, Zou S, Lan YL, Xing JS, Lan XQ, Zhang B. Solasonine inhibits glioma growth through anti-inflammatory pathways. Am J Transl Res. 2017 Sep 15;9(9):3977-3989.

[89]Zhang Y, Han G, Cao Y, Zhang Y, Zhang X, Gong H. Solasonine inhibits gastric cancer proliferation and enhances chemosensitivity through microRNA-486-5p. Am J Transl Res. 2020 Jul 15;12(7):3522-3530.

[90]Liu Z, Ma C, Tang X, Tang Q, Lou L, Yu Y, Zheng F, Wu J, Yang XB, Wang W, Hann SS. The Reciprocal Interaction Between LncRNA CCAT1 and miR-375-3p Contribute to the Downregulation of IRF5 Gene Expression by Solasonine in HepG2 Human Hepatocellular Carcinoma Cells. Front Oncol. 2019 Oct 18;9:1081. doi: 10.3389/fonc.2019.01081.

[91]Jin M, Shi C, Li T, Wu Y, Hu C, Huang G. Solasonine promotes ferroptosis of hepatoma carcinoma cells via glutathione peroxidase 4-induced destruction of the glutathione redox system. Biomed Pharmacother. 2020 Sep;129:110282. doi: 10.1016/j.biopha.2020.110282.

[92]Zhang X, Yan Z, Xu T, An Z, Chen W, Wang X, Huang M, Zhu F. Solamargine derived from Solanum nigrum induces apoptosis of human cholangiocarcinoma QBC939 cells. Oncol Lett. 2018 May;15(5):6329-6335. doi: 10.3892/ol.2018.8171.

[93]Al Sinani SS, Eltayeb EA, Coomber BL, Adham SA. Solamargine triggers cellular necrosis selectively in different types of human melanoma cancer cells through extrinsic lysosomal mitochondrial death pathway. Cancer Cell Int. 2016 Feb 17;16:11. doi: 10.1186/s12935-016-0287-4.

[94]Xie X, Zhu H, Yang H, Huang W, Wu Y, Wang Y, Luo Y, Wang D, Shao G. Solamargine triggers hepatoma cell death through apoptosis. Oncol Lett. 2015 Jul;10(1):168-174. doi: 10.3892/ol.2015.3194.

[95]Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol. 2000 Sep;10(9):369-77. doi: 10.1016/s0962-8924(00)01803-1.

[96]Breckenridge DG, Germain M, Mathai JP, Nguyen M, Shore GC. Regulation of apoptosis by endoplasmic reticulum pathways. Oncogene. 2003 Nov 24;22(53):8608-18. doi: 10.1038/sj.onc.1207108.

[97]Contassot E, Gaide O, French LE. Death receptors and apoptosis. Dermatol Clin. 2007 Oct;25(4):487-501, vii. doi: 10.1016/j.det.2007.06.010.

[98]Fan TJ, Han LH, Cong RS, Liang J. Caspase family proteases and apoptosis. Acta Biochim Biophys Sin (Shanghai). 2005 Nov;37(11):719-27. doi: 10.1111/j.1745-7270.2005.00108.x.

[99]Bruckheimer EM, Cho SH, Sarkiss M, Herrmann J, McDonnell TJ. The Bcl-2 gene family and apoptosis. Adv Biochem Eng Biotechnol. 1998;62:75-105. doi: 10.1007/BFb0102306.

[100]Blagosklonny MV. Paradox of Bcl-2 (and p53): why may apoptosis-regulating proteins be irrelevant to cell death? Bioessays. 2001 Oct;23(10):947-53. doi: 10.1002/bies.1135.

[101]Kucharczak J, Simmons MJ, Fan Y, Gélinas C. To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene. 2003 Dec 8;22(56):8961-82. doi: 10.1038/sj.onc.1207230. Erratum in: Oncogene. 2004 Nov 18;23(54):8858.

[102]Escárcega RO, Fuentes-Alexandro S, García-Carrasco M, Gatica A, Zamora A. The transcription factor nuclear factor-kappa B and cancer. Clin Oncol (R Coll Radiol). 2007 Mar;19(2):154-61. doi: 10.1016/j.clon.2006.11.013.

[103]Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci. 2005 Jan;30(1):43-52. doi: 10.1016/j.tibs.2004.11.009.

[104]Jana A, Krett NL, Guzman G, Khalid A, Ozden O, Staudacher JJ, Bauer J, Baik SH, Carroll T, Yazici C, Jung B. NFkB is essential for activin-induced colorectal cancer migration via upregulation of PI3K-MDM2 pathway. Oncotarget. 2017 Jun 6;8(23):37377-37393. doi: 10.18632/oncotarget.16343.

# Abbreviation

TCM: Traditional Chinese Medicine

5-Fu: 5-fluorouracil

GFs: Growth factors

JAK-STAT: Just another kinase

NFκB: Nuclear factor κB

mTOR: Mammalian target of rapamycin

BMP: Bone morphogenetic protein

MAPK: Mitogen-activated protein kinase

IBD: Inflammatory bowel disease

CEA: Carcino-embryonic antigen

FOLFOX: Oxaliplatin + 5-Fu + calcium linoleate regimen

XELOX: Capecitabine + oxaliplatin regimen

FOLFIRI: Irinotecan + 5-Fu + calcium linovolate regimen

DFS: Disease-free survival

OS: Overall survival

MDR: Multiple drug resistance

P-gp: P-glycoprotein

Caspase: Cysteine aspartic acid specific protease

MMP: Matrix metalloproteinases

EMT: Epithelial-mesenchymal transition

VEGF: Vascular Endothelial growth factor

NADPH: Nicotinamide adenine dinucleotide phosphate

DMSO: Dimethyl sulfoxide

FBS: Fetal bovine serum

DPBS: Dulbecco's phosphate-buffered saline

MTT: Thiazolyl blue tetrazolium bromide

PI: Propidium iodide

Bcl-2: B-cell lymphoma-2

BSA: Bovin serum albumin

AOD: IOD/area

Cyt-c: Cytochrome c

GPX4: Glutathione peroxidase 4

PARP: Poly ADP ribose polymerase

dATP: Deoxyadenosine triphosphate

IκBs: Cytoplasmic NFκB inhibitors

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