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Original article

Isorhamnetin in Tsoong blocks Hsp70 expression to promote apoptosis of colon cancer cells

Yunqi Luan [a,d], Yunpeng Luan [c,d], Youjie Zhao [b], Fei Xiong [b], Yanmei Li [c], Lili Liu [c], Yong Cao [b,1,*], Fei Dai [b,1,*]

[a] Soil System, Department of Geography and Ecology Lomonosov Moscow State University, Russia
[b] Research Institute of Big Data and Artificial Intelligence, Southwest Forestry University, Kunming 650224, PR China
[c] School of Life Science, Southwest Forestry University, Kunming, Yunnan 650224, PR China
[d] Key Laboratory for Forest Resources Conservation and Use in the Southwest Mountains of China, Ministry of Education, Southwest Forestry University, 650224, PR China

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ABSTRACT

The roots of *Codonopis bulleynana* Forest ex diels (*cb*Fed), locally known as Tsoong, have been used as a tonic food. Tsoong has wide range of pharmacological effects, including anticancer efficacy. In the present study, the anticancer activity of Tsoong and its potential molecular mechanisms were investigated. Isorhamnetin, a flavonol aglycone, is important compound and metabolite in Tsoong. It can promote apoptosis of colon cancer cells through up-regulating apoptosis-related genes (Apaf1, Casp3 and Casp9) because it blocks Hsp70 genes (Hspa1a, Hspa1b and Hspa8). These were verified by *in vitro* and *in vivo* experiments. *In vitro*, cell counting kit-8 (CCK-8) assays and flow cytometry in HCT116 and SW480 colon cancer cell were used to assess the anti-proliferation and apoptosis-promoting activities of Tsoong. *In vivo*, the antitumor effect of Tsoong was assessed in colon cancer-bearing nude mice as a xenograft model. These results show that Isorhamnetin is very critical in Tsoong because Tsoong can down-regulate Hsp70 genes and promote apoptosis of colon cancer cells by inhibiting Hsp70 largely due to the efficacy of Isorhamnetin. Our results may ultimately help in the development of diagnostic and therapeutic strategies to control this devastating disease.

1. Introduction

Colon cancer is one of the most common tumors worldwide. In recent decades, the epidemiological investigation has shown that due to the changes in living conditions and dietary structure, the incidence of colon cancer is gradually increasing in our country, especially in the young aged group, with an estimated 400 thousand new cases every year. Among them, colorectal cancer ranks the fourth highest in the incidence of malignant tumors and has risen to third in developed areas. Colon cancer has become one of the major diseases that endanger human health. Therefore, the mechanism of its occurrence and development as well as its therapeutic intervention have become major scientific issues in current oncology practices (Wang et al., 2014).

High fat diets, obesity, alcohol consumption, chronic inflammation of the gastrointestinal tract, and chronic constipation are considered to be the main causes of colorectal cancer (Liu et al., 2012; Joseph et al., 2013; Luan et al., 2016; Schulz et al., 2014; Anna et al., 2016). An enormous amount of researches have been concentrated upon the exact mechanism of colon cancer, and the effective prevention and treatment of colon cancer has become a focus of medical investigations (Katz et al., 2016).

Traditional Chinese medicine is appreciated for its 5000-year-old history and still holds an important position in primary health care in China, and it has also become more popular among cancer patients in the western world (Xin et al., 2012). In addition to traditional surgery, radiotherapy and chemotherapy, the combining of traditional Chinese medicine and Western medicine treatment strategies offer potential in the treatment of the colon cancer (Sommerer and Zeier, 2016; Liu and Liang, 2017). At present,

* Corresponding authors.
E-mail addresses: luanyunpeng2015@126.com (Y. Cao), cn_caoyong@126.com (F. Dai).
[1] These authors contributed equally to this work and should be considered co-corresponding author.
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natural medicine has become a focus of clinical anticancer drug investigation, due to its multi-target, multi-link and multi-channel antitumor effects.

Codonopsis (*Campanulaceae*, C.) is represented in China by 39 species, some of which are commonly used as herbal remedies due to their tonic effects, such as *C. pilosula* and *C. tangshen* (Hong et al., 1983). *Codonopis bulleynana* Forest ex diels (*cb*FeD) is herbaceous plant found in Yunnan, Tibet, and Sichuan Provinces. Its roots, locally known as Tsoong, have been used as a food in Yunnan Province since ancient times (Hu et al., 2012). Meanwhile, this species has become an important economic plant widely cultivated in several areas of Yunnan Province (Chen et al., 2006; Sun et al., 2016). Due to the characteristics of Tsoong being unique as a national medicinal herb, few reports exist in international journals, although there have been a wide range of investigations in China. Studies investigating the pharmacodynamics and acute toxicity of Tsoong have shown that it can enhance gastrointestinal peristalsis, improve tolerance to fatigue and hypoxia, and can promote the recovery of hemoglobin, red blood cells, IgG and the immunosuppressive effect in hemorrhagic blood deficient mice (Dong et al., 2015). Tsoong can enhance the immune function of mice with xenograft tumors and enhance the phagocytic functions of the reticuloendothelial system (Chen et al., 2012). It has been suggested that Tsoong has a positive effect on chemotherapy, reducing toxicity and enhancing immune function. The latest research literature shows that Tsoong can induce apoptosis and inhibits proliferation, migration and invasion of pancreatic ductal adenocarcinoma cells (Luan et al., 2018).

Caspases are a family of protease enzymes playing essential roles in apoptosis. Caspase deficiency has been identified as a cause of tumor development. Casp3 (Caspase3) and Casp6 (Caspase6) are the members of the Caspase family. They play central roles in the execution-phase of cell apoptosis. Casp3 and Casp6 can promote apoptosis in tumor cells and inhibit tumor (Han et al., 2017; Li et al., 2017; Suita et al., 2017; Suboj et al., 2012).

Apaf1 (Apoptotic protease activating factor 1) encodes a cytoplasmic protein that forms one of the central hubs in the apoptosis regulatory network. Apaf1 is one of the key regulators in the mitochondrial apoptotic pathway, and the loss of Apaf1 leads to cellular resistance against the apoptotic signals. Apaf1 plays an important role of in the development and progression of colorectal cancer (Seung et al., 2007).

Heat Shock Protein (HSP) can be divided into the following families according to the molecular weight: small molecule HSP (relative molecular mass, 22 ~ 32 ku), Hsp60, Hsp70, Hsp90, Hsp100 and ubiquitin (relative molecular mass, 7 ~ 8 ku), of which Hsp70 is the most important family with the largest expression amount after induction (https://en.wikipedia.org/wiki/Heat_shock_protein). Hsp70 is one of the most important members of HSP family, which is provided with the function of molecular chaperone, and involved in protein synthesis, processing, folding, transfer and other processes. Hsp70 can suppress the apoptosis-related genes such as Casp3 and Casp9 (Caspase9). Hsp70 are highly expressed in tumor cells, especially in malignant tumor cells. Simultaneously, it can play a role of anti-tumor cell apoptosis through gene regulation and immune response (Aghdassi et al., 2007).

Release of cytochrome c from mitochondria by apoptotic signals induces ATP/dATP-dependent formation of the oligomeric Apaf1–Casp9 (Caspase9) apoptosome. The documented anti-apoptotic effect of the principal heat-shock protein, Hsp70, is mediated through its direct association with the caspase-recruitment domain (CARD) of Apaf1 and through inhibition of apoptosome formation. The interaction between Hsp70 and Apaf1 prevents oligomerization of Apaf1 and association of Apaf1 with Procasp9 (procaspase9) (Ayman et al., 2000). It is shown that overexpression of Hsp70 can inhibit apoptosis-related genes and inhibit cell apoptosis.

Small molecule drugs are the focus in current anti-tumor researches. Quercetin is one of the most widely distributed flavonoid compounds in nature. Experimental studies show that quercetin can prevent the occurrence of tumors and significantly inhibit the growth of many malignant tumor cells and promote apoptosis. Quercetin has the effect of inducing apoptosis. The mechanism may be that the quercetin performed on tumor cells can lead to the downregulation of the expression of Hsp70. Isorhamnetin which is an O-methylated flavonol from the class of flavonoids, as a derivative of Quercetin, has the similar function of inhibiting Hsp70 (Gordana et al., 2002). Similarly, it can also inhibit the tumor (Teng et al., 2006; Prasain et al., 2016).

In present study, the possible effects and mechanisms of Tsoong against colon cancer was explored. Kunming mice were adopted to establish colon cancer model and Tsoong treatment model. Intestinal tissue samples from Kunming mice were used for high-throughput transcriptomic sequencing and the sequencing results were verified by qRT-PCR. The results showed that apoptosis-related genes were significantly down regulated in the disease model group of Kunming mice, and significantly increased in the Tsoong treatment group. The performance of Hsp70 genes was opposite of the apoptosis-related genes. Hsp70 were significantly up regulated in the disease model group of Kunming mice, and significantly decreased in the Tsoong treatment group. The results suggested that Tsoong can promote the apoptosis of colon cancer cells through suppressing Hsp70 and inhibit the intestinal cancer. Then *in vitro* and *in vivo* experiments were used to validate the inhibitory mechanism of Tsoong on intestinal cancer.

Loperamide acts on opioid receptors in the intestinal wall to inhibit the release of acetylcholine and prostaglandin, resulting in inhibition of intestinal peristalsis and prolonging of the retention time of intestinal contents, which lead to chronic constipation. Kunming mice were treated with Loperamide to establish chronic constipation model. After the successful establishment of constipation model the mice were continuously treated with Loperamide to establish colon cancer model. Anatomy showed that the mice with chronic constipation would have sarcomas in the intestines. Visible sarcomas under naked eyes were firstly found in the intestines of the colon cancer model group at the 9th week. There would be 6–12 tumors in the intestines in the 12th weeks. This meant that the colon cancer model was established successfully (Luan et al., 2017).

Oxaliplatin is a third-generation platinum anticancer drug, following cisplatin and carboplatin. Oxaliplatin induces autophagy and promotes the apoptosis of colon cancer cells (Tan et al., 2015). Oxaliplatin was used as control in the present study, in which the cytotoxic and antiproliferative effects of Tsoong on human colon cancer HCT116 and SW480 cell lines were examined. The present study also investigated apoptosis in HCT116 and SW480 cell in response to Tsoong treatment, and examined the consequences of Tsoong treatment using *in vivo* colon cancer models to obtain therapeutic insights.

2. Materials and methods

2.1. Establishment of colon cancer model and Tsoong treatment model

Materials. 72 Kunming mice were provided by Kunming Medical University. Main equipment for experiments included Leica stereomicroscope (Leica Company, Germany), optical microscope (Nikon Company, Japan), and microscopic imaging system (Nikon Company, Japan). Loperamide hydrochloride (Xian-Janssen Pharmaceutical Ltd., performance standard: YBH04562010,

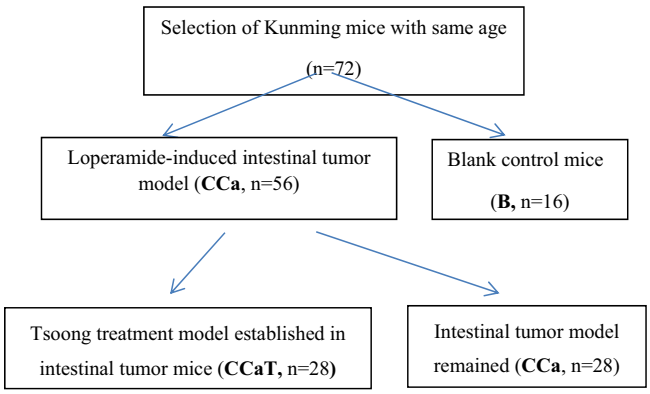

Fig. 1. Experimental flow chart. CCa stands for the experimental group gavaged with 1/2 original dosage of Loperamide to induce intestinal tumor after the successful establishment of constipation model (n = 56); **CCaT** stands for Tsoong treatment mice group in **CCa** (n = 28); The rest mice without Tsoong treatment remained **CCa** (n = 28).

specifications: 2 mg/tablet, batch number: 141111266), and all other needed regents were analytical reagents (Xilong Chemical Co., Ltd.).

Preparation of Loperamide. For constipation induction experiments, commercial Loperamide hydrochloride (2 mg/tablet) was dissolved in physiological saline to a concentration of 0.25 mg/mL, adjusted pH to 7.0, mixed well and stored at −20 for further use.

Experimental procedures. A total of 72 healthy Kunming mice with same age were screened and randomly divided into experimental group (**CCa**, n = 56) and blank control group (**B**, n = 16) (Fig. 1). For induction of constipation model, the **CCa** group was treated by intragastric administration of Loperamide according to the intragastric volume per unit of body weight of 2.5 mg/(Kg*d) for 2 consecutive weeks; the **B** group received intragastric administration with equal volume of sterile saline solution. Two weeks later, the constipation model was established successfully (Han et al., 2017).

Establishment of colon cancer model based on constipation model. After the successful establishment of constipation model, the **CCa** group with constipation were continuously treated with 1/2 original dosage Loperamide, which lead to colon cancer (Fig. 1). Stereo-microscope was used to observe lesion morphology, and lesions were fixed by 10% formalin solution and sliced into 5-μm sections according to the paraffin section method. Anatomy showed that the mice with chronic constipation would have sarcomas in the intestines. Visible sarcomas under naked eyes was firstly found in the intestines of the CCa group at the 9th week. There would be 6–12 tumors in the intestines in the 12th weeks. This meant that the colon cancer model was established successfully.

After the lesions in the intestines were fixed by 10% neutral formalin solution, sarcomas under the stereomicroscope presented uneven surface and abundant blood vessels in staggered and irregular arrangement (Fig. 2). Observation of pathological sections under high-power microscope revealed that cells showed irregular shape, inconsistent size, irregular nuclear shape, nuclear hyperchromatism, prominent nucleolus and nuclear division at lesion sites, which were confirmed as tumor cells when compared with relevant data (Fig. 3).

Establishment of Tsoong treatment model. After the successful establishment of colon cancer model, the **CCa** group was randomly divided into 2 groups. One group (**CCaT**, n = 28) was treated with Tsoong and the other group (**CCa**, n = 28) remained colon cancer model. After 12 weeks of Tsoong treatment, the tumors were markedly reduced. This meant that Tsoong treatment model was established successfully.

2.2. Metabolites in Tsoong extraction and detection

Standards and Reagents. All chemical reagents are analytically or chromatographically pure. The methanol, acetonitrile and ethanol were purchased from a German-based company, Merck KGaA.

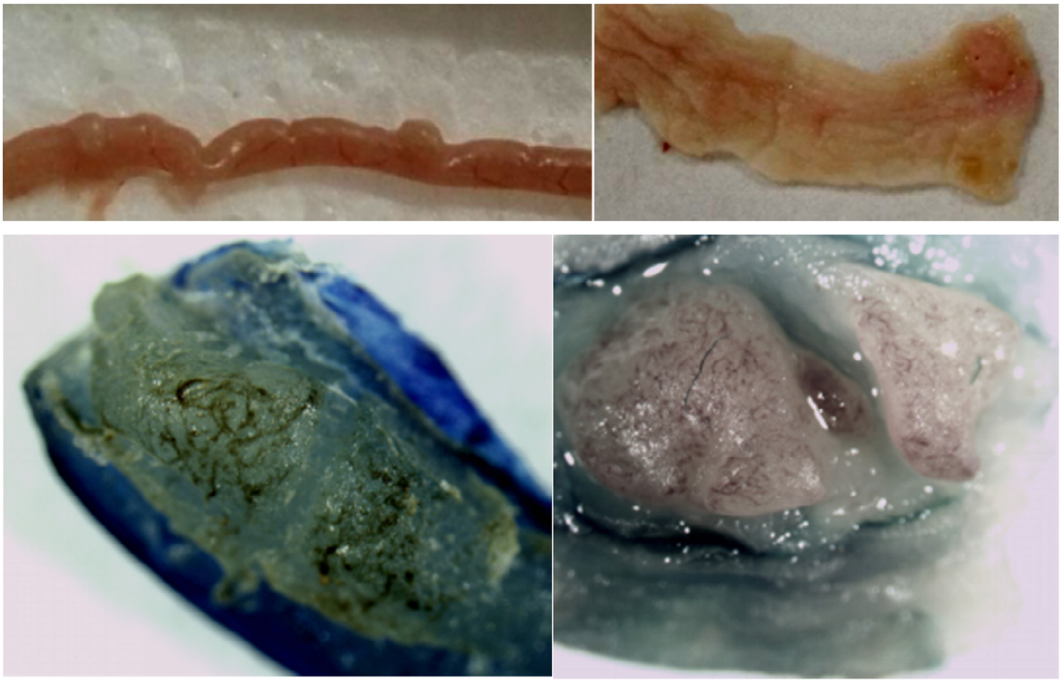

Fig. 2. Sarcoma morphology in the external and internal wall of the intestine (below images present visible sarcomas under naked eyes in the internal wall of the intestine under stereomicroscope after fixation by 10% neutral formalin solution).

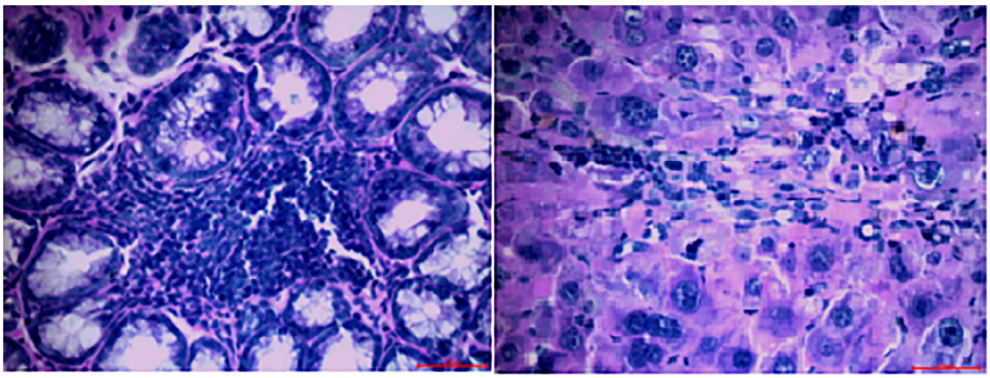

Fig. 3. Intestinal tumors under 40X microscope (H&E staining).

The water in use was double deionized ultrapure water obtained by a purification system purchased from Millipore (Millipore, Bedford, MA) (Zhao et al., 2018; Mehvish and Barkat, 2018). The chemical reference standards were purchased from BioBioPha and the American-based Sigma-Aldrich Corporation, and are dissolved in either dimethyl sulfoxide (DMSO) or methanol and stored at −20 °C before use. The working standards of the reference standards were diluted to different concentration gradients with 70% methanol before use for mass spectrometry analysis (Zhu et al., 2018; Alvi et al., 2018).

Metabolite extraction. The cryopreserved biomaterial specimens (slices of Tsoong) were vacuum lyophilized and ground with a grinder (MM 400, Retsch) at 30 Hz for 1.5 min. 100 mg of powder was weighed and then was extracted overnight at 4 °C using 1.0 ml of 70% methanol containing 0.1 mg/L lidocaine as internal standard. During the extraction, the sample was vortexed three times in order to make the extraction more thorough. After the extraction, the sample was centrifuged at 10,000 g for 10 min. The supernatant was collected and then filtered through filter membrane (pore size of 0.22 μm) and stored in a vial for LC-MS analysis.

Quality Control (QC) samples were prepared from sample extracts in order to examine the repeatability of samples under the same treatment method. In general, during the instrumental analysis process, one QC sample was inserted after every 10 tests in order to examine the repeatability of the analysis process (Fahim and Sathi, 2018; Bhuiyan et al., 2019).

Metabolite detection. The Data Acquisition Instrument System mainly consists of Ultra Performance Liquid Chromatography (UPLC) and Tandem Mass Spectrometry (MS/MS) (Applied Biosystems 4500 QTRAP). The main criteria for the UPLC analysis include: 1) Chromatographic column: Waters ACQUITY UPLC HSS T3 C18 1.8 μm, 2.1 mm*100 mm. 2) Mobile phase: ultrapure water (containing 0.04% acetic acid) as aqueous phase and acetonitrile (containing 0.04% acetic acid) as organic phase; 3) Elution gradient (water: acetonitrile): 95:5 V/V at 0 min, 5:95 V/V at 11.0 min, 5:95 V/V at 12.0 min, 95:5 V/V at 12.1 min, and 95:5 V/V at 15.0 min; 4) Flow rate: 0.4 ml/min; 5) Column temperature: 40 °C; 6) Injection volume: 5 μl. Separated samples enter the ESI-QTRAP-MS for mass spectrometry analysis.

In the API 4500 QTRAP LC/MS/MS system, the main parameters of linear ion trap and triple quadrupole are listed as follows: electrospray ionization (ESI) temperature of 550 °C, mass spectrometry voltage 5500 V, curtain gas (CUR) pressure of 25 psi, and a high collision-activated dissociation (CD) parameter. Within the triple quadrupole (QQQ), each ion pair was scanned under the optimized declustering potential (DP) and collision energy (CE). The data was processed using the software Analyst 1.6.1 (AB SCIEX).

2.3. Transcriptome sequencing

RNA extraction, library preparation, and next-generation sequencing. Next-generation sequencing is rapidly becoming the method of choice for transcriptional profiling experiments. Total RNA was isolated from snap-frozen colonic tissue from the control group and tumor samples from the experimental groups. using the AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA, USA). The quality and quantity of the extracted RNA samples were determined with bioanalyzer and ultramicro spectrophotometer, respectively. The library was constructed using the Illumina TruSeq RNA preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's manual. The next generation sequencing platforms most frequently used for RNA-seq are the Illumina HiSeq. Samples were sequenced on the Illumina instrument with 50–75 bp paired-end reads.

Analysis of RNA-seq. Raw Illumina fastq files were adapter-removed by software **cutadapt** (https://pypi.python.org/pypi/cutadapt/), Pollution-assessed by blast**+** (http://blast.ncbi.nlm.nih.gov/) and quality-controled by **Prinseq** (Schmieder and Edwards, 2011). The reads were aligned to the mouse genome with TopHat v2.0.10 (Kim et al., 2013) and The Cufflinks (Trapnell et al., 2010) program cuffdiff was used to calculate expression levels. Gene Ontology classification and enrichment analysis was performed base on the genes. KEGG PATHWAY is a collection of manually drawn pathway maps representing our knowledge on the molecular interaction and reaction networks for metabolism, genetic Information Processing, human Diseases and etc. (http://www.kegg.jp/). KEGG was used to annotate the genes.

Statistical analysis. Results were obtained by a variety of statistics and graphic software. The statistical analysis was performed with R packages, mothur (Schloss et al., 2009) and Samtools (http://samtools.sourceforge.net/). Welch's t-tests were conducted to compare the phenotypes of the different group mice and statistical significance was set at $P < 0.05$. To determine the role of the chronic constipation and chemical carcinogen in the development of colon tumorigenesis, the well-established model of constipation recapitulates the progression from chronic constipation to intestinal tumor in humans.

2.4. Validation of in vitro and in vivo experiments

qRT-PCR experiment verification. Through bioinformatics analysis the genes that may be related to tumor therapy had been selected. The apoptosis-related genes were screened out. They were Hspa8, Haspa1b, Casp3, Casp6 and Apaf1. The selected gene expressions in mice intestinal tissue was measured using qRT-PCR (Quantitative real time polymerase chain reaction). The following PCR primers were used as follow: Hspa8 forward 5′-CTGT

CCAGGCAGCCATTCTA-3′ and reverse 5′-AAGCAGGTTGTTGT CCTTGGTC-3′; Hspa1b forward 5′-GGTGAACTACAAGGGCGAGA-3′ and reverse 5′-GCTGAGAGTCGTTGAAGTAGGC-3′; Apaf1 forward 5′-AGATTTGGGATTCTGCGACTG-3′ and reverse 5′-GAAGGTGGT TACTCTTGTTGGTG-3′; Casp3 forward 5′-TGACTGGAAAGCC GAAACTC-3′ and reverse 5′-CAAGCCATCTCCTCATCAGTC-3′; Casp6 forward 5′-CGATTGCTTCATCTGTGTCTTC-3′ and reverse 5′-GTCTC CTTTGAACAAGCCAGTC-3′.

Fluorescence PCR amplifications were performed with ABI step one plus. The qRT-PCR reactions were performed in triplicate for the three biological replicates. Relative quantification was performed using the $2^{-\triangle\triangle Ct}$ method, including an efficiency correction for the primers using the Relative Expression Software Tool.

Cell culture. The HCT116 and SW480 cell lines were obtained from American Type culture collection (Manassas, VA, USA). The HCT116 and SW480 colon cancer cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (cat. no. 10099158; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml of streptomycin and 100 U/ml of penicillin in a humidified atmosphere of 5% CO_2 at 37 °C.

Preparation of drug-containing serum. The dry Tsoong was purchased from Yunnan International Pty, Ltd. (Yunnan, china). Firstly, 1 kg of dry Tsoong was soaked in cold water for 30 min, decocting twice with 1:6 w/v distilled water for 1 h. Filtration was then performed to the appropriate concentrations, the first decoction comprised in 1:10 w/v distilled water for 90 min and second comprised 1:8 w/v distilled water for 60 min. A final quantity of 450 g dried powder was obtained by spray drying at room temperature, which was then sealed and stored in the dark at 4 °C. The Tsoong powder was dissolved in normal saline for the gavage experiments. The drug-containing serum solutions were collected from mice following exposure to the following treatments (once per day for 1 week): Treatment with normal saline by gastrogavage (n = 8; normal control group); 5 g/kg of Tsoong by gastrogavage (n = 8; low Tsoong group); 10 g/kg of Tsoong by gastrogavage (n = 8; mid Tsoong group), 20 g/kg of Tsoong by gastrogavage (n = 8; high Tsoong group); 5 mg/kg oxaliplatin by gastrogavage (n = 8; oxaliplatin group). The blood samples were obtained from the abdominal aorta following treatment, following which the serum was isolated by centrifuging at 1,800 × g for 10 min at 4 °C and stored at −80 °C for the follow-up experiments.

Experimental groups. The drug-containing serum solutions from the mice were used to treat the HCT116 and SW480 cells. The five treatment groups comprised the normal control group, the low Tsoong group, the mid Tsoong group, the high TSoong group, and the oxaliplatin group.

Cell counting kit-8 (CCK-8) assay. cell proliferation was determined using the colorimetric water-soluble tetrazolium salt assay using a CCK-8 kit (Beyotime Institute of Biotechnology, co., Ltd., Haimen, china). In brief, the cells at a density of 2×10^3 cells per well was seeded in 96-well plates and incubated with the low, mid, or high dose of Tsoong, with or without oxaliplatin, for 24, 48 and 72 h. Following treatment, the culture medium was removed and replaced with 100 μl of fresh medium containing 10 μl of CCK-8 solution in each well and the cells were incubated at 37 °C for 2 h. The number of viable cells was determined by reading the absorbance at 450 nm using a Thermo Platemicroplate reader (Rayto Life and Analytical Science co., Ltd., Shenzhen, 103 china).

Cell cycle assay. Following treatment, the cells were harvested and resuspended at a density of 1×10^6 cells/ml. The cells were fixed with ice-cold 70% ethanol for at least 30 min. cell cycle was analyzed using a flow cytometer with propidium iodide (PI) as a specific fluorescent dye probe. The PI fluorescence intensity of 10,000 cells was measured for each sample using a Becton-dickinson FAcScalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell apoptosis assay. Following treatment, cell apoptosis was assessed using an Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). In brief, Annexin V-FITC (5 μl) and PI (5 μl) were added to 100 μl cells at a concentration of 1×10^6 cells/ml, and incubated in the dark for 15 min at room temperature. Subsequently, binding buffer was added and apoptosis was analyzed using ow cytometry (BD Biosciences).

Xenograft tumors. A total of 18 female Balb/c athymic nude mice (5–6 weeks old, body weight 19–22 g) (Vital River Laboratories, Beijing, China) were housed at 25 °C in 40–70% humidity, in a 12 h light/dark cycle with free access to food and water and were subcutaneously injected in the right flank with 2.0×10^6 SW480 cells in 0.1 ml PBS. When tumors had formed, the tumor volume (V) was measured using calipers daily and calculated using the following formula: $V=(LxW^2)/2$, where L was the length and W was the width of the tumor. The mice were randomly divided into three groups (n = 5): Normal control group mice treated with normal saline via gavage; high dose of Tsoong (20 g/kg) mice treated with a high dose of Tsoong via gavage; oxaliplatin group mice with colon cancer treated with oxaliplatin (5 mg/kg via gavage). Growth curves were plotted using the average tumor volume within each experimental group every week. After six weeks, the mice were sacrificed, and the dissected tumors were collected and prepared for subsequent analyses. All animal experiments were approved by the Animal center of Southwest Forestry University (Kunming, china). All experimental procedures involving animals were performed according to the institutional ethical guidelines for animal experiments and in accordance with the Guide for the care and Use of Laboratory Animals.

Statistical analysis. The results are presented as the mean ± standard deviation. The statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). For comparisons Dunnett *t*-test or two-way analysis of variance was used. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Analysis of transcriptome sequencing

Effects of Tsoong on the apoptosis-related genes. The correlation between the gene expression of samples is an important indicator to reflect the reliability and the rationality of sample selection. In order to facilitate the comparison of different experimental groups, the **B** was used to indicate blank group, the **CCa** was used to indicate colon cancer group, and the **CCaT** was used to indicate Tsoong treatment group. The differential gene test method uses **cuffdif2** in **Cufflink**. The software is widely used in the RNA-seq analysis with the reference genome. Filter condition was set to p-Value < 0.05. In organisms, different genes coordinate their biological functions. The most important biochemical and signal transduction pathways of differentially expressed genes are identified by pathway enrichment. KEGG (Kyoto Encyclopedia of Genes and Genomes) is the main public database of pathway.

Through the comparison of **B** vs. **CCa** and **CCa** vs. **CCaT**, 272 DEGs and 178 DEGs were identified, respectively. To distinguish the most affected pathways, a KEGG enrichment analysis (corrected p-Value < 0.001 and FDR < 0.001) was performed. The significantly enriched KEGG pathways in **B** vs. **CCa** are shown in Fig. 4.

It can be seen in Fig. 4 **Apoptosis** pathway and **Longevity regulating** pathway were among the top 20 significantly enriched KEGG pathways about colon cancer. Therefore, **Apoptosis** pathway and **Longevity regulating** pathway were very important KEGG

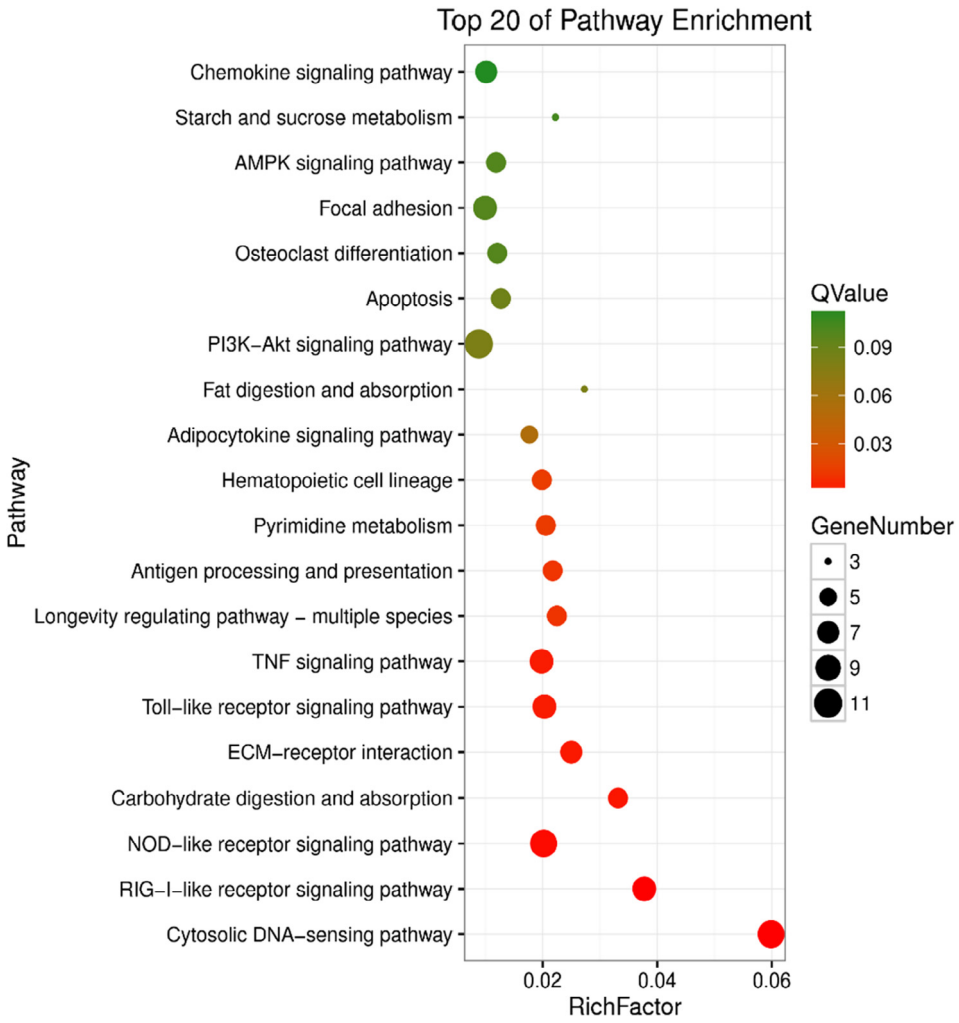

Fig. 4. The significantly enriched KEGG pathways in B vs. CCa. Apoptosis pathway was one of the most significantly enriched KEGG pathways.

Table 1
The selected genes in **Apoptosis** pathway in **B** vs. **CCa.**

Gene	foldChange	pvalue	B_fpkm	CCa_fpkm
Casp3	0.500	0.015	27.07	13.51
Casp6	0.858	0.602	118.65	101.76
Apaf1	0.255	0.020	12.64	3.21

Table 2
The selected genes in **Apoptosis** pathway in **CCa** vs. **CCaT.**

Gene	foldChange	pvalue	CCa_fpkm	CCaT_fpkm
Casp3	1.100	0.699	13.51	14.99
Casp6	1.040	0.602	101.766	105.4
Apaf1	1.542	0.02	3.21	4.94

Table 3
The selected genes in **Longevity regulating** pathway in **B** vs. **CCa.**

Gene	foldChange	pvalue	B_fpkm	CCa_fpkm
Hspa1a	2.411	0.047	7.14	17.24
Hspa1b	3.580	0.004	8.70	31.11
Hspa8	4.365	0.000	13.57	59.27

Table 4
The selected genes in **Longevity regulating** pathway in **CCa** vs. **CCaT.**

Gene	foldChange	pvalue	CCa_fpkm	CCaT_fpkm
Hspa1a	0.113	0.000	17.24	1.95
Hspa1b	0.096	0.000	31.11	3.00
Hspa8	0.447	0.000	59.27	26.48

pathways in disease development. Some important selected genes in **Apoptosis** pathway are shown in Table.1 and Table.2. They are Casp3 (Caspase3), Casp6 (Caspase6) and Apaf1. The other important selected genes in **Longevity regulating** pathway are shown in Table.3 and Table.4. They are Hspa1a, Hspa1b, and Hspa8.

Heat shock 70 kDa protein 8 known as heat shock cognate 71 kDa protein or Hsc70 or Hsp73 is a heat shock protein that in humans is encoded by the Hspa8 gene on chromosome 11 (https://en.wikipedia.org/wiki/HSPA8). Heat shock 70 kDa protein 1, also termed Hsp72, is a protein that in humans is encoded by the Hspa1a gene (https://en.wikipedia.org/wiki/HSPA1A). Heat shock 70 kDa protein 1B, also known as Hspa1b, is a human gene (https://en.wikipedia.org/wiki/HSPA1B). As the members of the heat shock protein 70 family (Hsp70) and a chaperone protein, they facilitate the proper folding of newly translated and misfolded proteins, as well as stabilize or degrade mutant proteins. They have been associated with an extensive number of cancers, neurodegenerative diseases, cell senescence, and aging [42, 43, 44]. Hsp70 family

are molecular chaperones that play essential roles in tumor growth by stabilizing pro-survival client proteins. From literature it can be known that Hsp70 plays a critical part in regulating mitochondrial apoptotic pathway and apoptosis-related genes (Ayman et al., 2000).

Verification to Effects of Tsoong treatment using qRT-PCR Experiments. Because some data did not meet the requirements (p-value < 0.05) the qRT-PCR were conducted to verify the high throughput sequencing results. Differentially expressions of the above 3 genes in **B** group, **CCa** group and **CCaT** group were studied with qRT-PCR (quantitative real-time Reverse Transcription Polymerase Chain Reaction) experiments to verify the above results and efficacy of Tsoong. The qRT-PCR results are shown in Fig. 5. Through high throughput sequencing and qRT-PCR experiment verification it can be seen that these apoptosis-related genes (Casp3, Casp6 and Apaf1)were downregulated in the colon cancer model group (**CCa**) and they were obviously recovered in the Tsoong treatment group (**CCaT**).

Differentially expressions of Hsp70 were verified by qRT-PCR in **B** group, **CCa** group and **CCaT** group in the same way and the Hsp70 included Hspa1b and Hspa8. The qRT-PCR results are shown in Fig. 6. Through high throughput sequencing and qRT-PCR experiment verification it can be seen that Hsp70(Hspa1a, Hspa1b and Hspa8)were upregulated very high in the colon cancer model group (**CCa**) and they were obviously downregulated very low in the Tsoong treatment group (**CCaT**).

3.2. Metabolites in Tsoong evaluation and analysis

Based on the local self-built metabolite database, the metabolites of samples were qualitatively analyzed by mass spectrometry. The analysis of metabolite structure was referred to MassBank (http://www.massbank.jp/), KNAPSAcK (http://kanaya.naist.jp/KNApSAcK/), HMDB (http://www.hmdb.ca/), MoToDB (http://www.ab.wur.nl/moto/) and METLIN (http://metlin.scripps.edu/index.php) etc. The metabolite profile of the extract determined by LC-MS is represented in Fig. 7. The MRM (multiple-reaction monitoring) metabolite detection multiple mass spectral peak map showed the material that can be detected in the sample, and the mass spectra peak of each different color represented a detected metabolite. LC-MS analysis revealed presence of Isorhamnetin (Rt: 5.91) in Tsoong.

The molecular structure of Isorhamnetin is shown as Fig. 8. It is an O-methylated flavonol from the class of flavonoids.

3.3. In vitro experiments

Effects of Tsoong on the proliferation and cell cycle of HCT116 117 and SW480 cells. *Codonopsis* can induce cell cycle arrest and apoptosis in human colon tumor (Wang et al., 2013). *cb*Fed is a species of the *Codonopsis* and Tsoong is the root of *cb*Fed. The Tsoong-containing serum solutions were prepared from mice by

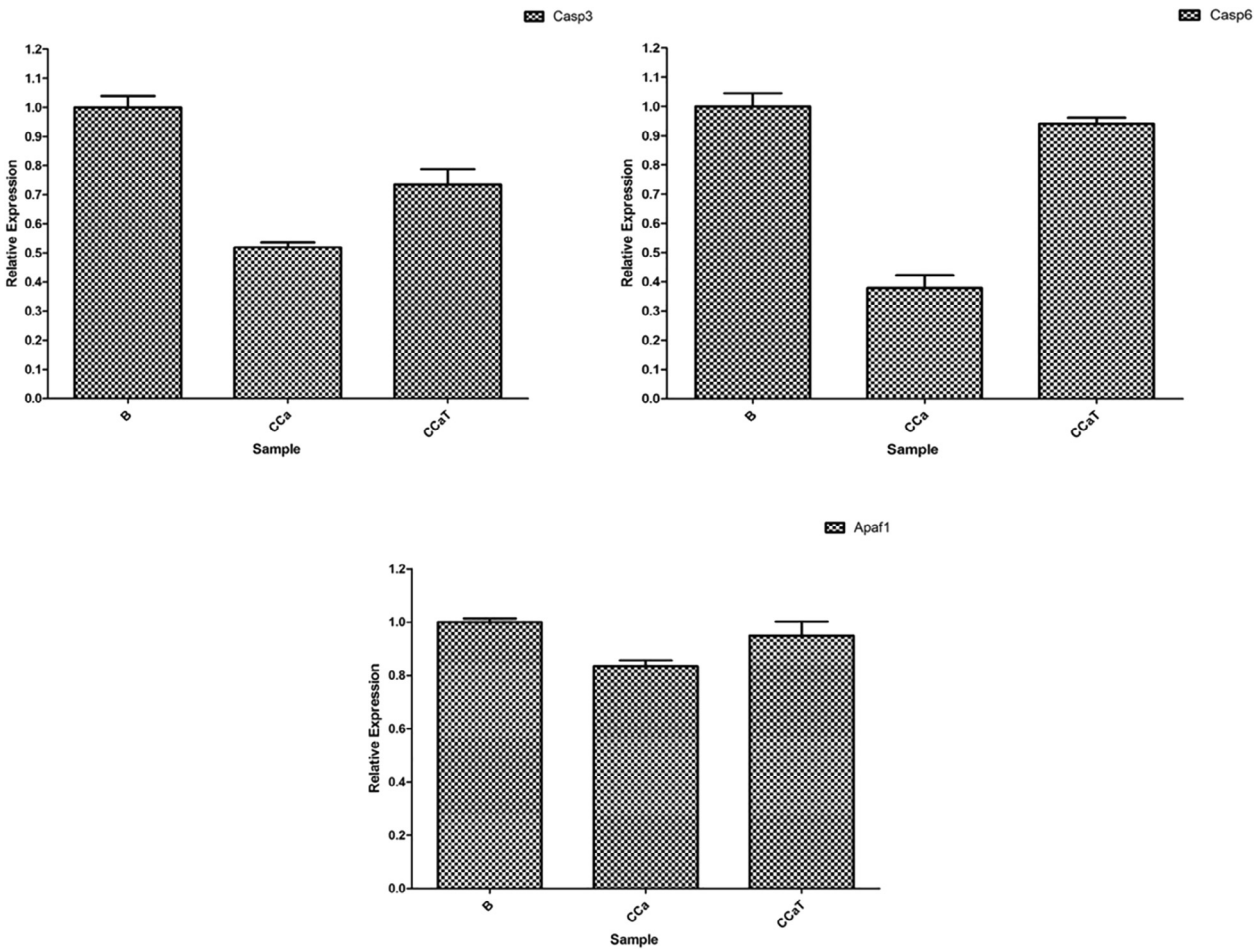

Fig. 5. Differentially expression of Casp3, Casp6 and Apaf1 in B group, CCa group and CCaT group were verified with qRT-PCR experiments. They are the most important apoptosis-related genes. The qRT-PCR experiments verified that the Tsoong restored the expression of apoptosis genes.

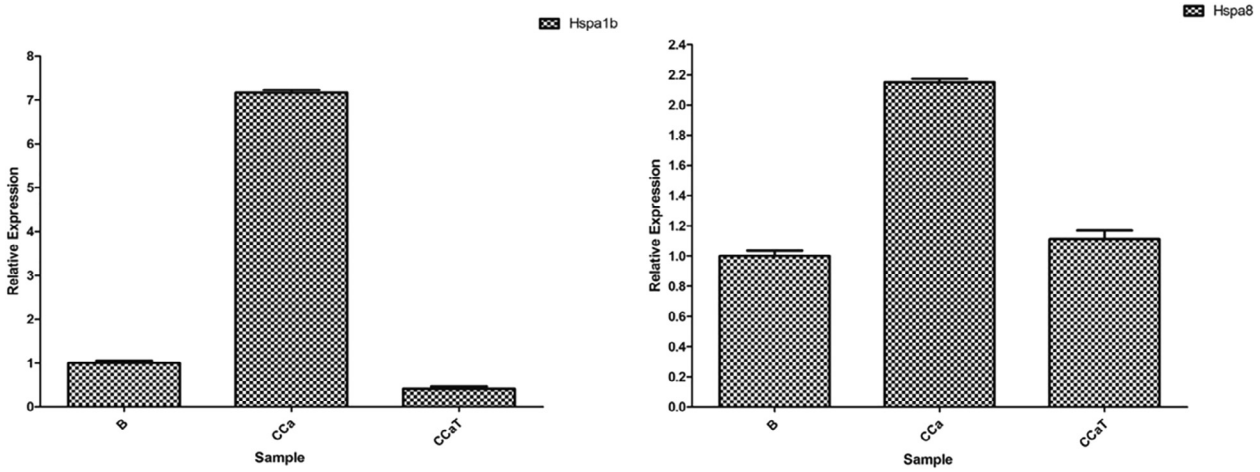

Fig. 6. Differentially expression of Hspa1b and Hspa8 in B group, CCa group and CCaT group were verified with qRT-PCR experiments. They are critical genes in regulating mitochondrial apoptotic pathway and apoptosis-related genes. The qRT-PCR experiments verified that the Tsoong suppressed the expression of Hsp70 genes greatly.

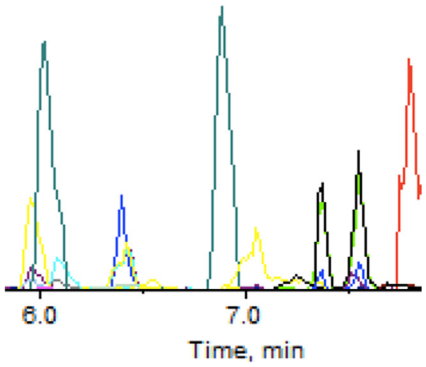

Fig. 7. LC-MS analysis revealed presence of Isorhamnetin (Rt: 5.91) in Tsoong.

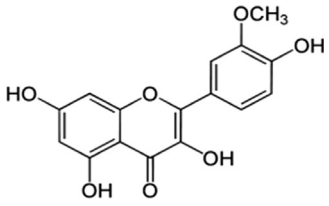

Fig. 8. The molecular structure of Isorhamnetin. It is an O-methylated flavonol.

gastrogavage with saline (control), or 5 (low), 10 (mid) and 20 (high) g/kg of Tsoong. To determine the role of Tsoong on cell proliferation, cell cycle and cell apoptosis, the HCT116 and SW480 cells were treated with these Tsoong-containing serum solutions. The results showed that Tsoong inhibited the proliferation of HCT116 and SW480 cells at 48 and 72 h. The cell proliferation rate was decreased with increasing concentrations of Tsoong-containing serum solutions, suggesting that Tsoong inhibited the cell proliferation in a dose-dependent manner (Fig. 9A). The cell proliferation in the high Tsoong group was similar to that in the oxaliplatin group. Similarly, Tsoong decreased the proportion of cells in the G1 phase cells but increased the proportion of cells in the S phase, suggesting that Tsoong induced S phase arrest (Fig. 9B), which was similar to the effect of oxaliplatin. Therefore, Tsoong inhibited cell proliferation in a dose-dependent manner and induced cell cycle arrest at the S phase.

Effects of Tsoong on the apoptosis of HCT116 and SW480 cells. The apoptotic rates of HCT116 and SW480 cells following Tsoong treatment were also analyzed (Fig. 7C). The apoptotic rates of the HCT116 cells treated with low, mid, and high doses of Tsoong were 16.6, 27.4 and 32.7%, respectively, whereas the apoptotic rate of the HCT116 cells in the control group was only 6.2%. Tsoong treatment produced the same effects on the SW480 cells. The results suggested that the apoptotic rate induced by Tsoong was increased with dose. Treatment of the cells with oxaliplatin confirmed the apoptosis of the HCT116 and SW480 cells.

3.4. In vivo experiments

Tsoong suppresses tumorigenicity in vivo. To confirrm the above findings, particularly the results of the CCK-8 assay (Fig. 9A), and due to the fact that SW480 cells have been used in the establishment of xenograft tumors in previous studies (Wang et al., 2011; Chen et al., 2016), SW480 cell were used to establish a nude-mouse transplanted tumor model in the present study. A high dose of Tsoong or oxaliplatin were administered to nude mice by gastrogavage or injection, 6 weeks following intragastric administration, these two groups exhibited significantly smaller tumors, compared with those in the normal saline group (Fig. 10A and B). The H&E staining showed that the efficacy of Tsoong was similar to that of oxaliplatin. (Fig. 10C).

4. Discussion

There are many kinds of proteases in caspase family, which can be stepwise hydrolyzed and activated and promote the progress of apoptosis jointly. The pro-apoptosis factor Casp3 is one of the most important members of the cysteine protease family, and it exists in cytoplasm in the form of an inactive zymogen. The original structural domain of Casp3 will be cleaved off by proteolysis under the stimulation of apoptotic signals and be activated through the formation of a tetramer. The activated Casp3 participates in a series of cell change processes related to apoptosis, including the degradation of intracellular protein substrates, inactivation of apoptosis inhibitors, aggregation of chromatin and promotion of apoptotic body formation. Casp6 gene is an effector molecule of Caspase and is the most important apoptotic executor besides Casp3. It turns into an active form only after the activation of the upstream Caspase. The large and small subunits aggregate to form a dimer, while two dimers can be polymerized into a more active tetramer then, which acts on substrates related to the downstream apoptosis and causes apoptosis (Jiang et al., 2017; Braton et al., 2000).

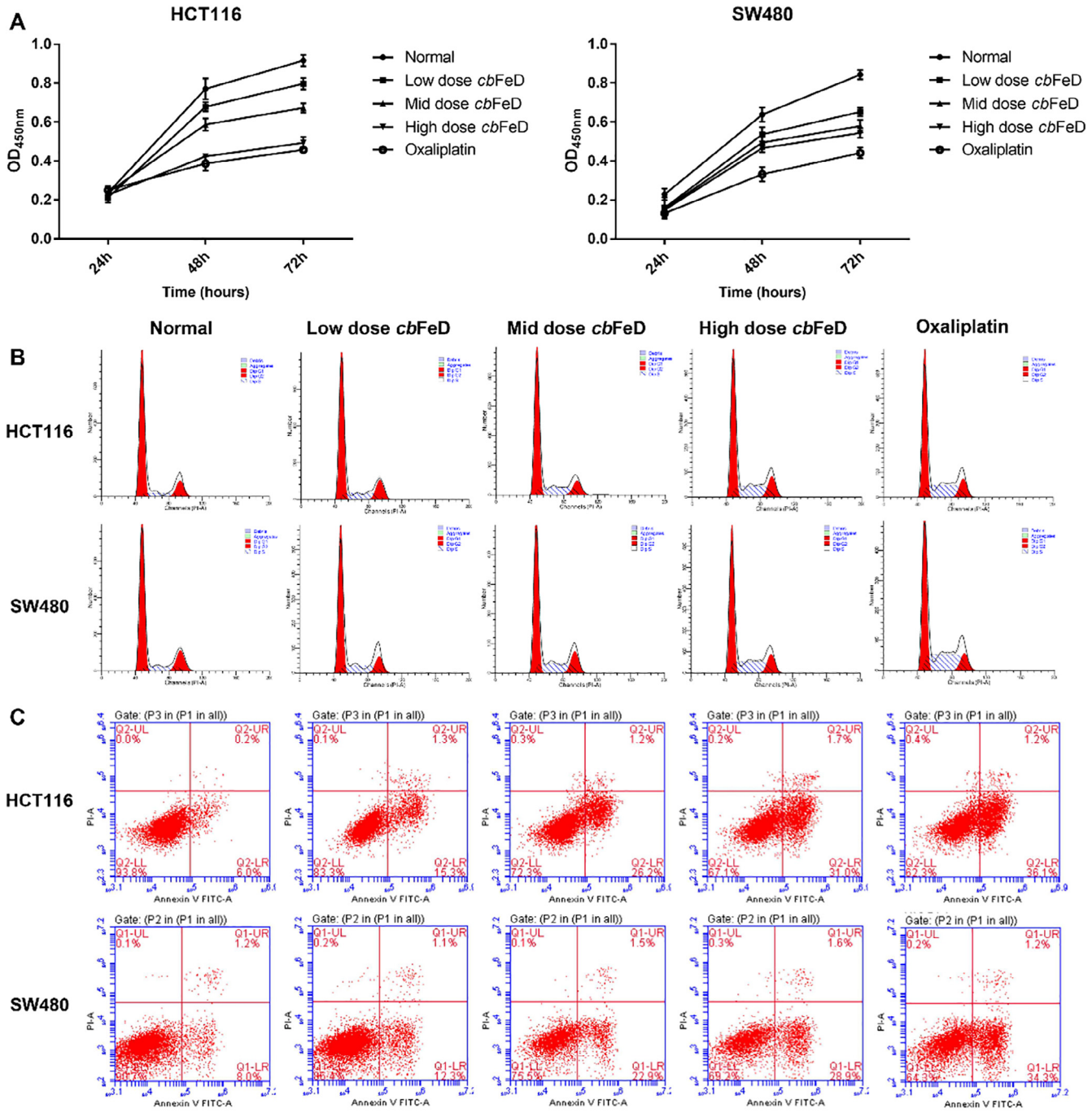

Fig. 9. Effect of Tsoong on the proliferation and cell cycle of HCT116 and SW480 cancer cells. The cells were treated with Tsoong-containing serum solutions prepared from mice treated by gastrogavage with saline (control), or 5 (low), 10 (mid) or 20 (high) g/kg of Tsoong for indicated durations. (A) cell proliferation was detected using a cell counting kit-8 assay, and (B) cell cycle was detected using flow cytometry. (C) Cell apoptosis was analyzed using flow cytometry. Oxaliplatin was used as a control. *P < 0.05 vs. control; **P < 0.01 vs. control. Tsoong or *cb*FeD, *Codonopis bulleynana* Forest ex diels.

Apaf1 plays a pivotal role in cellular apoptosis and is involved in the mitochondria-mediated apoptosis pathway. Different forms of cellular stress induce the release of cytochrome *c* from the gap of mitochondrial inner membrane into the cytoplasm directly. With the presence of dATP/ATP, cytochrome *c* binds to Apaf1 in the cytoplasm and recruits procasp9(procaspase9) after the oligomerization of Apaf1, thus an apoptotic body will be formed, in which procasp9 is activated into Casp9 (Caspase9) before the activation of Casp3 (Caspase3) and other proteins in caspase family by the apoptotic body, leading to irreversible apoptosis. It is obvious that Apaf1 is an important pro-apoptotic factor in the mitochondrial apoptotic pathway as well as the core of the apoptotic body (Bossy-Wetzel and Green, 1999; Zlobec et al., 2007; Strater et al., 2010; Goldstein et al., 2000).

Heat shock proteins (Hsps) are overexpressed in a wide range of human cancers and are implicated in tumor cell proliferation, differentiation, invasion, metastasis, death, and recognition by the immune system. In cancer, high levels of Heat shock protein 70 (Hsp70) are associated with poor prognosis and resistance to chemotherapeutics (Ciocca and Calderwood, 2005). Rohde M et al. revealed in their experiment that HSP70 could prevent the differentiation of cancer cells in G2/M and G2 phases, respectively.

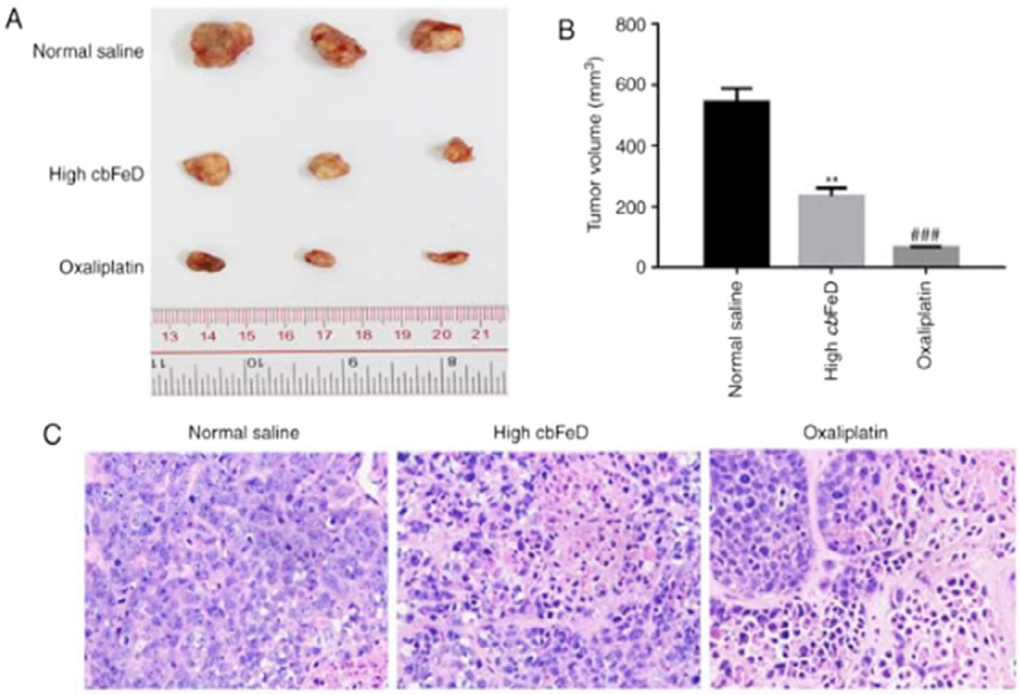

Fig. 10. Effects of Tsoong and oxaliplatin on growth of xenograft tumors. Nude mice were subcutaneously injected in the right flank with 2.0×10^6 SW480 cells in 0.1 ml PBS. Once tumors had formed, tumor volume was measured. The mice were then randomly divided into three groups (n = 5): Normal control group, mice treated with normal saline via gavage; high Tsoong group, mice treated with a high dose (20 g/kg) of Tsoong via gavage; oxaliplatin group, mice with colon cancer treated with oxaliplatin (5 mg/kg) via gavage or injection. After 6 weeks, the dissected tumors were collected and H&E were performed. (A) Tumor size. (B) tumor volume. (C) H&E staining of xenograft tumors. Magnification, x400. Oxaliplatin was used as a control. *P < 0.01 vs. control; **P < 0.001 vs. control. Tsoong or *cb*FeD, *Codonopis bulleynana* Forest ex diels.

By contrast, the absence of Hsp70 could result in the arrested growth or apoptosis of cells (Rohde et al., 2005). Meanwhile, another experiment conducted by Daugaard M et al. also showed that Hsp70 was essential for the survival and growth of tumor cells (Daugaard et al., 2005).

Cytochrome c can connect with apoptotic protease-activating factor 1 (Apaf1) after released from mitochondria, and activate Casp9 in ATP/dATP dependent condition. Caspase family is the intracellular core regulator of apoptosis, of which Casp3 is the major trigger of apoptosis. The activated Caspase has the ability to activate authigenic proenzyme and Casp3, thus amplifying the cascade reaction of Caspase. Meanwhile, Casp9 and Apaf1 are the key to cascade activation of Caspase, and the two are connected together by Caspase activating and recruitment domain (CARD). Hsp70 can combine with Apaf1 directly, or hinder the exposure of Apaf1 CARD area, and inhibit Casp9 proenzyme gathering to apoptotic body, thus preventing the formation of apoptotic body. Therefore, Hsp70 can induce the inactivation of Casp9 by competing the CARD structure of Apaf1 with Casp9, thereby affecting the cascade reaction of Caspase within cytoplasm, further suppressing the activation of Casp3 and others, and inhibiting cell apoptosis eventually (Ayman et al., 2000).

The transcription of Hsp70 will be greatly enhanced in tumor cells basically, such as in stomach cancer, cervical cancer, ovarian cancer, and etc. A large number of prior experiments have documented that highly expressed Hsp70 can protect tumor cells. High expression of Hsp70 can inhibit tumor cell death and generate tolerance in tumor cells. With respect to the above, it is speculated that if the expression of Hsp70 can be reduced by inhibiting the transcription of mRNA, it will effectively kill the tumor cells, which can provide a new approach for cancer gene therapy.

Natural products have been used in traditional and folk medicine for therapeutic purposes. They provide important sources of promising leads for the development of novel therapeutic drugs (Wang et al., 2011). The fresh roots of *Codonopis bulleynana* Forest ex Diels, locally known as Tsoong, have been used as a food or vegetable and herbal remedies due to their tonic effects in Yunnan Province since ancient times (Chen et al., 2006). Recently, phytochemical research also revealed that the crude extract of Tsoong increased the chemotherapeutic sensitivities and attenuated and enhance immune function of tumor-bearing mice (Chen et al., 2012).

Apoptosis is essential for cell development and tissue homeostasis in eukaryotic organisms. Susceptibility of mammalian cells to apoptosis is determined by a balance between the activities of pro-apoptotic and anti-apoptotic molecules. Apoptotic process has become one of the targets for cancer treatment because of its frequent loss in various tumors and contribution to chemotherapy and radiotherapy resistance [56]. Tumorigenesis and development is closely related to aberrant cell apoptosis process. Therefore, apoptosis is believed to play a significant role in tumorigenesis. In particular, induction of apoptosis is the main mechanism of action for anti-cancer drugs. The ability and potency for a particular drug compound to induce apoptosis also becomes an important dimension for its evaluation. Apoptosis-related genes (e.g., Casp3, Casp6 and Apaf1) can largely determine whether a cell would enter apoptosis process.

Therefore, Apaf1, Casp3, Casp6 and so on are the most important genes involved in apoptosis. Hsp70 are very critical in regulating apoptotic-related genes or proteins. In the mouse disease model group, apoptotic-related genes were significantly down-regulated, followed by a significant rebounded after Tsoong treatment. On the contrary Hsp70 were significantly up-regulated in the colon cancer model group, and significantly recovered in the Tsoong treatment group. It not only was a result of high-throughput transcriptome sequencing, but also was verified by qRT-PCR experiment. Then, *in vitro* and *in vivo* experiments had further demonstrated that Tsoong could promote tumor cell apoptosis and inhibit intestinal cancer.

Tsoong has wide range of pharmacological effects, including anticancer effects. The anticancer role of Tsoong was confirmed in the present study. In this study Tsoong can suppress Hsp70 greatly, while Hsp70 can prevent mitochondrial apoptotic pathway and inhibit apoptosis genes. Hence Hsp70 can protect tumor cells and promote them proliferation and metastasis. Therefore, Tsoong can inhibit cell proliferation and metastasis by suppressing Hsp70. These were verified by *in vivo* and *in vitro* experiments. Through *in vitro* experiments of colon cancer cell HCT116 and SW480 Tsoong has been proven that it can increase the proportion of cells in the S phase, and promote the apoptosis of tumor cells. The inhibition of cell proliferation, and promotion of cell cycle arrest and cell apoptosis in the HCT116 and SW480 cells by Tsoong were found to occur in a dose-dependent manner. Similarly, through *in vivo* experiments of nude mice xenograft Tsoong has been confirmed that it can inhibit the growth of colon cancer and induce the apoptosis of xenograft tumor cells.

Flavonoids and Flavonols belong to a very vast group of plant secondary metabolites with variable phenolic structures and are found in almost every part of the plant. Many epidemiological studies have been conducted to prove the protective effect of flavonoids against cancer. They have been reported that compounds such as Quercetin and Isorhamnetin from different plant sources have efficacy against different cancer. Isorhamnetin, an essential monomer derived from total flavones, has also shown a spectrum of antitumor activity. Its mechanisms of action may involve apoptosis of cells induced by down-regulation of oncogenes and up-regulation of apoptotic genes (Li et al., 2015; Teng et al., 2006; Ramachandran et al., 2012; Fei and Wei, 2018; Lee et al., 2008). Flavonoids and flavonols such as derivatives of Quercetin are rich in the Tsoong.

Through LC-MS analysis Isorhamnetin is important compound or metabolite of Tsoong. Isorhamnetin is a flavanoid present in plants and is also an immediate metabolite of quercetin in mammals. The plasma level of isorhamnetin is maintained longer than quercetin and isorhamnetin may be a key metabolite to mediate the antitumor effect of quercetin. Isorhamnetin can augment sub-G1 apoptotic portion significantly. Western blot analyses revealed increased cleavage of Casp3, and Casp9 and PARP and increased cytosolic cytochrome C in isorhamnetin-treated cells. These events were accompanied by a reduced mitochondrial potential. Cytochrome C release in isorhamnetin-treated cells was a likely consequence of a loss of mitochondrial mem- brane potential. These results support Isorhamnetin induced mitochondria-dependent caspase activation cascade and mitochondria-dependent caspase activation to mediate isorhamnetin-induced apoptosis. Therefore, Isorhamnetin can significantly increase supporting a mitochondria-dependent pathway for caspase-mediated apoptosis (Lee et al., 2008).

Quercetin is a known specific inhibitor of Hsp70 synthesis and thus might be a potent agent for enhancing the selective cytotoxicity of heat on tumour cells. Structurally related flavonoids and flavonols especially Isorhamnetin can also suppress the expression of Hsp70 significantly. Isorhamnetin has the effect of inducing apoptosis. The mechanism may be that the Isorhamnetin perform on tumor cells can lead to the downregulation of the expression of Hsp70 and Hsp90 as well as the genetic expression of Bcl-2, thus inhibiting the genetic expression related to proliferation, promoting the release of cytochrome C from mitochondria, prevent the combination of Hsp70 and Apaf1, promoting the formation of tumor cells apoptosomes and inducing the increase of Casp3 enzymatic activity in tumor cells [26, 61]. Because Isorhamnetin is important component of Tsoong and Isorhamnetin can block expressions of Hsp70 significantly, Tsoong inhibits Hsp70 and promotes apoptosis of tumor cells largely due to the critical role of Isorhamnetin.

5. Conclusion

In conclusion, the present study demonstrated that: (i) Isorhamnetin is important component and metabolite in Tsoong; (ii) Tsoong can significantly suppress expression of Hsp70 genes which inhabited apoptosis-related genes and prevented tumor cells; (iii) Tsoong can significantly promote expression of apoptosis-related genes; (iiii) Tsoong can inhibit proliferation and promote apoptosis of colon cancer cells and restrain the growth of colon neoplasm in nude mouse. These results show that Isorhamnetin is very important in Tsoong because Tsoong can down-regulate Hsp70 genes expressions and promote apoptosis of colon cancer cells by blocking Hsp70 largely due to the efficacy of Isorhamnetin. Our results may ultimately help in the development of diagnostic and therapeutic strategies to control this devastating disease. Therefore, these results indicated that Tsoong may be a potential candidate for the prevention of colon cancer and a promising Chinese herbal compound for development for use in cancer therapy.

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Compliance with ethical standards.

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

The sampling of human peritoneal tissue was approved by the Local Ethics Committee (Yunnan Medical University, KMMU: 2017004), in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Further reading

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