Available online at www.sciencedirect.com
ScienceDirect
Chinese Journal of Natural Medicines 2022, **20**(1): 9-21
doi: 10.1016/S1875-5364(21)60118-8
Chinese Journal of Natural Medicines

•Research article•

β-Elemene induces apoptosis and autophagy in colorectal cancer cells through regulating the ROS/AMPK/mTOR pathway

WANG Guo-Yu[1, 2, 3Δ], ZHANG Lei[1, 2Δ], GENG Ya-Di[1], WANG Bin[2], FENG Xiao-Jun[1], CHEN Zhao-Lin[1], WEI Wei[2*], JIANG Ling[1, 2*]

[1] *Department of Pharmacy, the First Affiliated Hospital of USTC, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei, Anhui, 230001,China;*
[2] *Institute of Clinical Pharmacology, Anhui Medical University, Hefei 230001, China;*
[3] *Department of Pharmacy, Hefei Cancer Hospital, Chinese Academy of Sciences, Hefei 230031, China*

Available online 20 Jan., 2022

[ABSTRACT] *β*-Elemene is an effective anti-cancer ingredient extracted from the genus *Curcuma* (Zingiberaceae familiy). In the present study, we demonstrated that *β*-elemene inhibited the proliferation of colorectal cancer cells and induced cell cycle arrest in the G_2/M phase. In addition, *β*-elemene induced nuclear chromatin condensation and cell membrane phosphatidylserine eversion, decreased cell mitochondrial membrane potential, and promoted the cleavage of caspase-3, caspase-9 and PARP proteins, indicating apoptosis in colorectal cancer cells. At the same time, *β*-elemene induced autophagy response, and the treated cells showed autophagic vesicle bilayer membrane structure, which was accompanied by up-regulation of the expression of LC3B and SQSTM1. Furthermore, *β*-elemene increased ROS levels in colorectal cancer cells, promoted phosphorylation of AMPK protein, and inhibited mTOR protein phosphorylation. In the experiments *in vivo*, *β*-elemene inhibited the tumor size and induced apoptosis and autophagy in nude mice. In summary, *β*-elemene inhibited the occurrence and development of colon cancer xenografts in nude mice, and significantly induced apoptosis and autophagy in colorectal cancer cells *in vitro*. These effects were associated with regulation of the ROS/AMPK/mTOR signaling. We offered a molecular basis for the development of *β*-elemene as a promising anti-tumor drug candidate for colorectal cancer.

[KEY WORDS] *β*-Elemene; Colorectal cancer; Apoptosis; Autophagy; AMPK; mTOR
[CLC Number] R965 **[Document code]** A **[Article ID]** 2095-6975(2022)01-0009-13

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in many Asian and European countries [1]. In China, CRC has ranked the fifth in the incidence of common cancers and related death [2]. Although surgical resection is the primary curative management for the early stage of CRC, a growing number of patients are diagnosed at the advanced stage due to lack of reliable and effective screening. Chemotherapy still serves as a common therapeutic strategy for CRC. Unfortunately, many patients eventually relapse, and chemotherapy resistance occurs [3-4]. Therefore, there is an urgent need to develop effective and low-toxic antitumor drugs for the treatment of CRC.

Programmed cell death (PCD) is probably the most commonly studied subject in the field of cancer therapy. PCD plays a key role in both carcinogenesis and cancer treatment. Apoptosis, also known as type I PCD, is characterized by specific morphological changes of dying cells, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to the neighbours or to extracellular matrix [5]. Autophagy (type II PCD) is an evolutionarily conserved catabolic process beginning with formation of autophagosomes, double membrane-bound structures surrounding cytoplasmic macromolecules and organelles, destined for recycling [6-7]. In general, autophagy plays a crucial pro-survival role in cell homeostasis, and is required during starvation or stress due to growth factor deprivation [8]. However, there is accumulating evidence that

[Received on] 11-Jan.-2021
[Research funding] This work was supported by the National Natural Science Foundation of China (Nos. 81603339 and 81803774), Anhui Key Research and Development Program (No. 201904a07020092), the Fundamental Research Funds for the Central Universities (Nos. WK9110000016 and WK9110000079), and China Postdoctoral Science Foundation (No. 2019M662207).
[*Corresponding author] Tel:86-551-65161206, E-mail: wwei@ahmu.edu.cn(WEI Wei); Tel: 86-551-65592671, E-mail: jiangling3339@163.com (JIANG Ling)
ΔThese authors contributed equally to this work.
These authors have no conflict of interest to declare.

autophagic cells may commit suicide by undergoing cell death or executing apoptotic pathway [9]. Apoptosis and autophagy may jointly decide the fate of cancer cells.

The root stalk of the genus *Curcuma* as a traditional Chinese herbal medicine is a commonly used drug for promoting blood circulation and removing blood stasis [10-11], from which *β*-elemene is extracted and has been demonstrated to be a new type of anti-tumor drug candidate [12]. A large number of studies [13-14] have shown that *β*-elemene exhibits anti-tumor effects on various cancers such as liver cancer, gastric cancer, breast cancer and brain cancer. Its preparation has been approved as a chemotherapy-assisted drug for clinical cancer treatment in China [15]. However, the effect of *β*-elemene on CRC is little known. The current study was aimed to investigate how *β*-elemene affected CRC *in vitro* and *in vivo*. The obtained results suggested *β*-elemene as a promising drug candidate with unique molecule mechanism against CRC.

Materials and Methods

Reagents and antibodies

β-Elemene (purity > 95%) was purchased from Dalian Jingang Pharmaceutical Co., Ltd. (Dalian, China). *β*-Elemene was dissolved in dimethyl sulfoxide (DMSO) for all experiments in this study. The primary antibodies to cleaved-caspase-3, caspase-3, cleaved-caspase-9, caspase-9, cleaved-PARP, PARP, LC3B, SQSTM1, p-AMPK, AMPK, p-mTOR, mTOR and Ki-67 were purchased from Cell Signaling Technology (Danvers, USA). The primary antibody to GAPDH was obtained from Beijing Bioss Biotechnology Co., Ltd. (Beijing, China).

Cell culture

Human CRC cell lines DLD-1 and HT-29 were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). DLD-1 and HT-29 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DME/F12) supplemented with 10% fetal bovine serum (FBS), 100 $U \cdot mL^{-1}$ penicillin and 100 $mg \cdot mL^{-1}$ streptomycin. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C and passaged every two to three days.

MTS assay

The effects of *β*-elemene on the proliferation of DLD-1 and HT-29 cells were assessed using MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2*H*-tetrazolium] assay. DLD-1 and HT-29 cells in the logarithmic growth phase were seeded into 96-well plates at 200 μL per well, with a total of 5000 cells. After the cells were grown to 50% to 60% confluence, they were exposed to serum-free medium and *β*-elemene at different concentrations for 24, 48, and 72 h, respectively. The morphology of cells in each group was observed under an microscope. After treatment, MTS (at a final concentration of 333 $\mu g \cdot mL^{-1}$)/PMS (25 $nmol \cdot L^{-1}$) was added, and the cells were incubated at 37 °C for 1 h. A microplate was used to measure the optical density (*OD*) values at 490 nm. The results were expressed as cell viability (%) = (*OD* of the drug group/*OD* of the blank control group) × 100. Triplicate experiments were performed for each concentration and the results are presented as mean ± SD.

Clone formation assay

DLD-1 and HT-29 cells at the logarithmic growth phase were seeded into 6-well plates at 2 mL per well (1 × 1000 cells). After 24 h, the cells were exposed to serum-free medium and *β*-elemene at different concentrations for 24 h. Then the drug solution was discarded, and 2 mL of DMEM containing 10% fetal bovine serum was added to each well, and the culture medium was changed every two days. Cell colonies were grown and processed after 14 days. Then, the cells were washed twice with PBS, before exposed to 1 mL paraformaldehyde for 30 min, to which 1 mL of filtered crystal violet solution was added for staining over 15 min. Then, the crystal violet was removed, and the cells were gently rinsed with water and allowed to air dry prior to imaging.

Flow cytometry analysis of cell cycle

Distribution of cell cycle was determined by flow cytometry. Briefly, cells were seeded into 6-well plates at a density of 1×10^5 cells/well and grown to 60%−70% confluency, before exposure to serum-free medium and *β*-elemene at different concentrations for 24 h. Then, the cells were harvested and fixed, and the cell cycle was detected by the cell cycle detection kit (Shanghai Beibo Biological Co., Ltd., Shanghai, China) according to the manufacturer's insturctuions. Percentages of cells within each cell cycle compartment (G_0/G_1, S, and G_2/M) were determined by flow cytometry (CytoFLEX, Beckman Coulter).

Hoechst 33342 staining

DLD-1 and HT-29 cells were seeded into 12-well plates at a density of 1×10^5 cells/well. After 24 h, the cells were exposed to serum-free medium and *β*-elemene at different concentrations for 24 h. Then, the cells were washed with PBS, to which 1 mL of Hoechst 33342 staining solution was added in each well before incubation for 15 min. The staining solution was then discarded, and the cells were washed with PBS twice and observed under a fluorescence microscope.

Flow cytometry analysis of cell membrane phosphatidylserine eversion

DLD-1 and HT-29 cells were treated with serum-free medium and *β*-elemene at different concentrations for 24 h. Cell membrane phosphatidylserine eversion rates were determined by flow cytometry using the Annexin V-FITC/PI double staining cell apoptosis detection kit (Shanghai Beibo Biological Co., Ltd., Shanghai, China) according to the manufacturer's insturctuions. The left lower quadrant of each plot indicates the survival cells. The right lower quadrant indicates the apoptotic cells and the right upper quadrant indicates the necrotic cells. Their percentages were determined by flow cytometry (CytoFLEX, Beckman Coulter). Results were from

triplicate experiments.

Flow cytometry analysis of cell mitochondrial membrane potential

DLD-1 and HT-29 cells were treated with serum-free medium and *β*-elemene at different concentrations for 24 h. Cell mitochondrial membrane potential was determined by flow cytometry using the apoptosis mitochondrial membrane potential detection kit (JC-1) (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) according to the manufacturer's instructions. The mitochondrial transmembrane potential is depolarized during apoptosis, and JC-1 is released from the mitochondria as a monomer in the cytoplasm. Based on this feature, the changes in mitochondrial membrane potential were detected. Mitochondrial membrane potential was determined by flow cytometry (CytoFLEX, Beckman Coulter).

AO staining for detection of acidic organelles

DLD-1 and HT-29 cells were treated with *β*-elemene at different concentrations and serum-free medium for 24 h. The cells were exposed to 1 μg·mL^{-1} AO (acridine orange) staining solution in darkness for 10 min, and washed three times with cold PBS. The fluorescence intensity of acidic organelles was determined by flow cytometry (CytoFLEX, Beckman Coulter).

Transmission electron microscopy for detecting apoptosis and autophagosomes

DLD-1 and HT-29 cells were treated with *β*-elemene at different concentrations and serum-free medium for 24 h. The cells were harvested and fixed with 1 mL glutaraldehyde. The cell pellet was embedded in 4% agar, cuted into pieces of 1 mm^3, fixed with 1% citrate for 1.5 h, and dehydrated with ethanol at different concentrations (30%, 50%, 70%, 90%, and 100%), replaced with propylene oxide. Dehydrated samples were treated with Epon-812 resin and acetone, and polymerized in a polymerizer, and the polymerized samples were embedded and sectioned under a solid microscope. The ultrathin sections were double-stained with uranyl acetate-lead citrate. Photographs were taken under a transmission electron microscope.

DCFH-DA staining detecting ROS levels

DLD-1 and HT-29 cells were treated with *β*-elemene at different concentrations and serum-free medium for 24 h. ROS levels were determined with a Reactive Oxygen Species Assay Kit (Shanghai Beyotime Biotech Co., Ltd., Shanghai, China) according to the manufacturer's instructions. Afterward, ROS levels were analyzed using a fluorescence microscope (DMI3000B, Germany Lycra). At least five fields were randomly selected and images were taken from each well.

Western blot

DLD-1 and HT-29 cells were treated with *β*-elemene at different concentrations and serum-free medium for 24 h. The total protein was extracted from each group. Protein concentration was determined by BCA method and the protein was adjusted to the same amount. The proteins were transferred to PVDF membranes by SDS-PAGE method, and blocked with 5% skim milk at room temperature for 2 h. The membranes were then incubated with primary antibodies at 1 ∶ 1000 dilution with 5% BSA in PBST at 4 °C overnight. The blots were washed and incubated with HRP-conjugated secondary antibodies (1 ∶ 10 000, Bioworld, USA) at room temperature for 1 h. The membranes were visualized using enhanced chemiluminescence (Immobilon ECL, Millipore, USA).

Colon cancer nude mouse model

Mice were strictly maintained according to the requirements of the Animal Ethics Committee of the First Affiliated Hospital of USTC and the National Institutes of Health (NIH) standard guidelines for the Care and Use of Laboratory Animals. HT-29 tumor inoculation (4×10^6 cells at the right hind limb) was performed on 6-week old BALB/c male mice. The successfully modeled nude mice were obtained after five days. The mice were divided randomly into five groups (n=6): a normal group (unmodeled), a model group, a low-dose *β*-elemene group (25 mg·kg^{-1}), a high-dose *β*-elemene group (50 mg·kg^{-1}), and a positive control 5-FU group (25 mg·kg^{-1}). The animals were intraperitoneally injected with the corresponding agnets for 21 days. Mice in *β*-elemene treatment groups were administered once daily from Monday to Friday; the positive control 5-FU group was administered once every other day; and the normal group and model group were given the same volume of normal saline. Body weight was measured once every two days. On day 25, all mice were sacrificed, and the tumor was collected, weighed, and stored in −80 °C for later use. Tumor volume (TV, mm^3) was calculated as $TV = ab^2/2$, where a is the longest diameter and b is the shortest diameter. Tumor inhibitory ratio IR (%) was calculated as IR (%) = (1 − WT/WC) × 100, where WT and WC are the average tumor weights of the treated and control groups, respectively.

For histological examinations, 5 mm-thick sections of tumor tissues in paraffin blocks were stained with hematoxylin and eosin (H&E). *β*-Elemene-induced apoptosis was evaluated by TUNEL assay using the *in situ* cell death detection kit (Roche, USA) according to the manufacturer's instructions. To assess cell proliferation, Ki-67 assay was performed. Immunohistochemical staining was conducted to measure the expression of Ki-67, cleaved-caspase-3 and LC3B, according to the manufacturer's instructions (Beijing Zhongshang Jinqiao Biotechnology Co., Ltd. Beijing, China). Images were obatined under a fluorescence microscope (DMI3000B, Germany Lycra).

Statistical analysis

Data were statistically analyzed by SPSS 16.0 software. All experiments were repeated for three times or more. Comparison of multiple data sets was performed using one-way analysis of variance (ONE-WAY ANOVA). $P < 0.05$ was considered significance.

Results

β-Elemene reduces cell viability and induces cell cycle arrest at the G_2/M phase in human CRC cells

We initially evaluated the effects of *β*-elemene on the vi-

ability of DLD-1 and HT-29 cells. MTS assay showed that *β*-elemene reduced the viability of DLD-1 cells in both concentration- and time-dependent manners (Fig. 1A). Similar results were recaptured in HT-29 cells (Fig. 1A). The results of colony formation experiments showed that *β*-elemene concentration-dependently reduced the number of clones of HT-29 cells (Fig. 1B). We also observed that *β*-elemene induced cell cycle arrest in the two cell lines evidenced by flow cytometry analysis with quantification. *β*-Elemene at 100 μmol·L^{-1} significantly arrested the cell cycle at the S checkpoint and at 200 μmol·L^{-1} more significantly arrested the cell cycle at the G_2/M phase in DLD-1 cells (Fig. 2A). *β*-Elemene at 200 μmol·L^{-1} more significantly arrested the cell cycle at the G_2/M phase in HT-29 cells (Fig. 2B). These results indicated that *β*-elemene inhibited cell viability and induced cell cycle arrest at the G_2/M phase in CRC cells.

β-Elemene induces apoptosis in human CRC cells

To examine the molecular mechanism involved in *β*-elemene's effects on CRC cells, we performed a series of experiments focusing on apoptosis. Results in Fig. 3A showed that *β*-elemene-treated DLD-1 and HT-29 cells exhibited fragmented nucleus, in which the nuclear chromatin was concentrated and bright and dense compared with the control cells (Fig. 3A). Electron microscopy analysis showed that *β*-elemene-treated cells showed intrachromosomal chromatin condensation, cleavage, and marginalization, which were supportive for apoptosis (Fig. 3B). Flow cytometry analysis demonstrated that *β*-elemene dramatically triggered phosphatidylserine eversion in the cell membrane of HT-29 cells (Fig. 4A). In addition, *β*-elemene decreased the mitochondrial membrane potential (△Ψm) in the two lines of CRC cells, and the number of cells in early apoptosis significantly increased (Fig. 4B). Furthermore, Western blot analysis revealed that *β*-elemene increased the levels of cleaved-caspase-3, cleaved-caspase-9, and cleaved-PARP in the two lines of CRC cells (Fig. 5). Collectively, these findings implied that *β*-elemene induced apoptosis in human CRC cells.

β-Elemene enhances autophagy through activating the ROS/AMPK/mTOR pathway in human CRC cells

Autophagy achieves the metabolic needs of the cells themselves and some organelle renewals, which can be encountered in both the physiological and pathological processes of the body [6]. Here, a series of experiments were performed to assess the effects of *β*-elemene on autophagy in DLD-1 and HT-29 cells. First, we observed intracellular autophagic vacuoles in the cells under an electron microscope. As shown in Fig. 6A, *β*-elemene-treated HT-29 cells contained a significant number of vesicle bilayer membrane structure and autophagic vacuoles compared with the control cells (Fig. 6A). Consistently, AO staining assay showed that the acidic vesicle organelles were formed in *β*-elemene-treated HT-29 cells, which is a characteristic of autophagy (Fig. 6B). Next, Western blot analysis was used to detect the activation of LC3B and SQSTM1, two marker proteins of autophagy, and found that *β*-elemene increased the protein levels of LC3B-II and SQSTM1 concentration-dependently in the two CRC cell lines (Fig. 6C). We next explored the molecular pathways underlying *β*-elemene-enhanced autophagy. It is

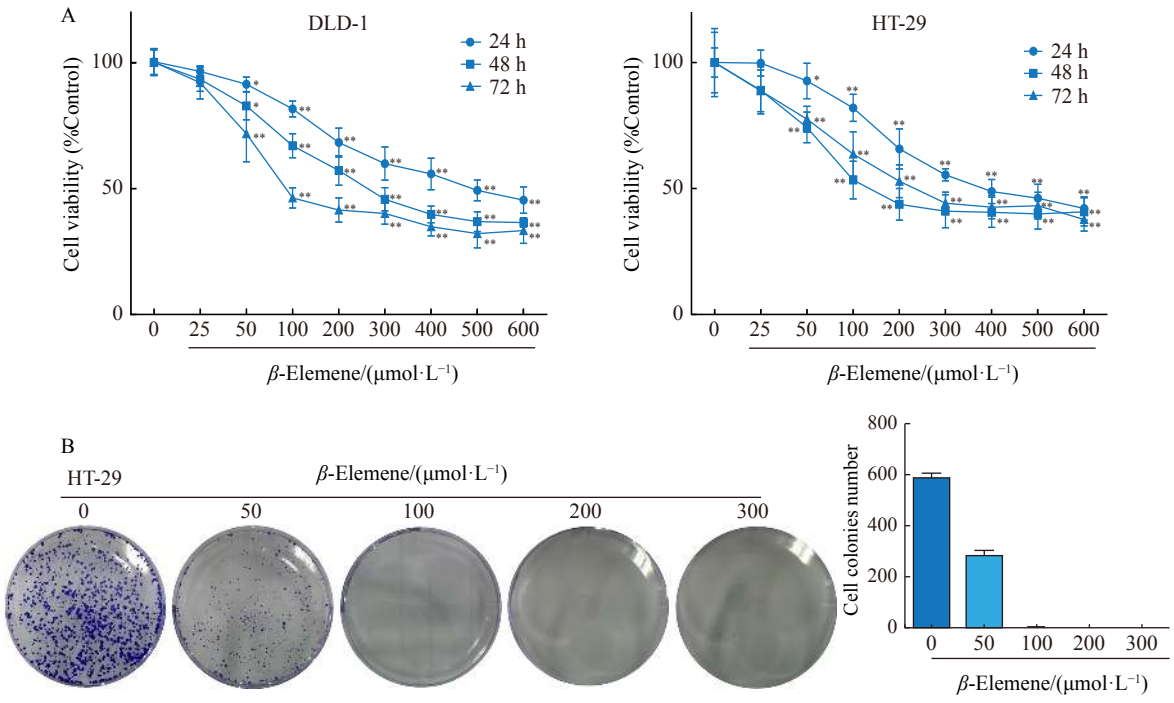

Fig. 1 *β*-Elemene inhibits the proliferation of human CRC cells. (A) Human CRC cell viability after *β*-elemene treatment was determined by MTS assay. (B) Representative pictures of colony-forming assay and number of HT-29 cell colonies. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the untreated control. The experiment was repeated at least three times.

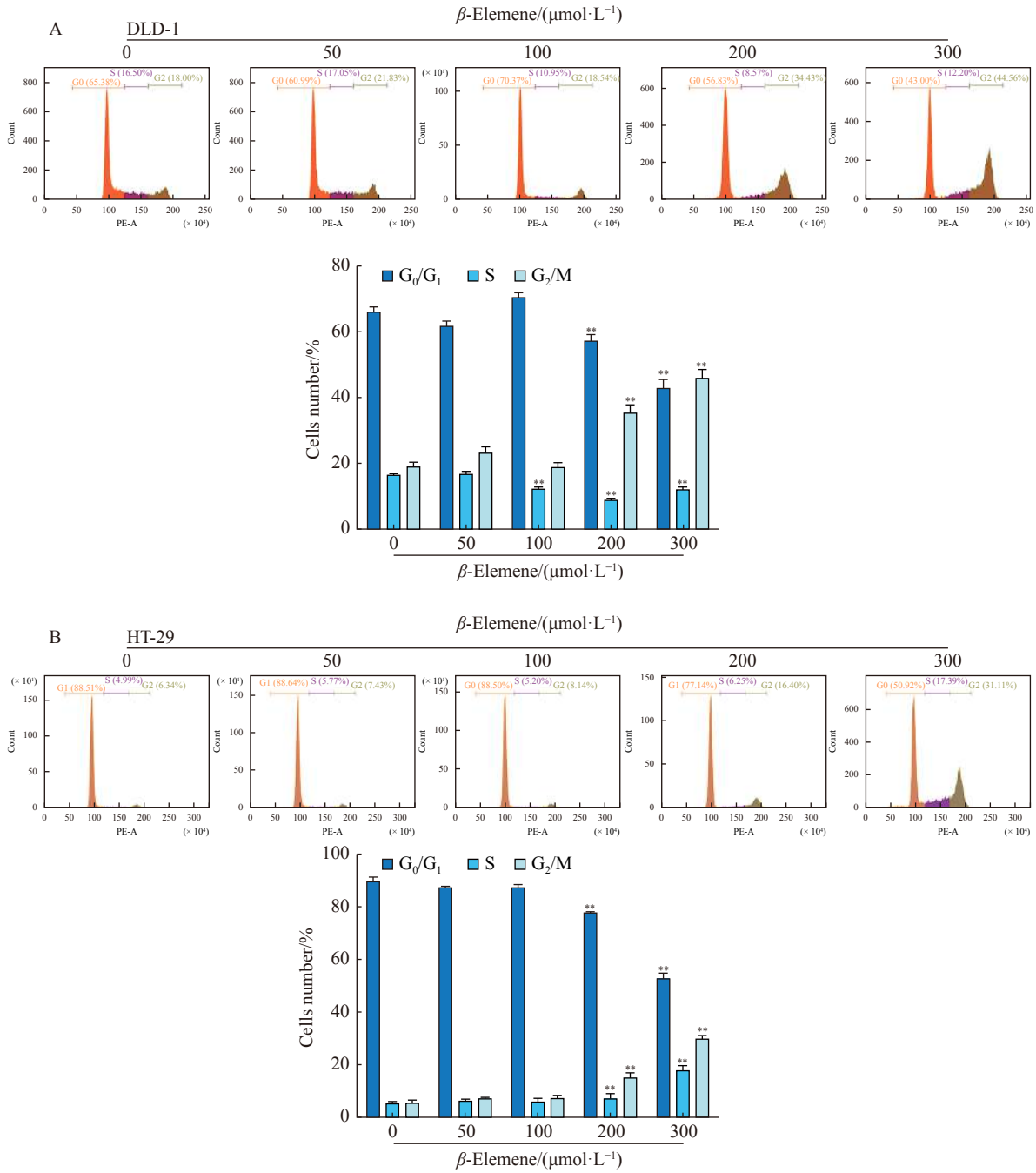

Fig. 2 *β*-Elemene regulates the cell cycle of human CRC cells. (A) The cell cycle of DLD-1 cells was detected by flow cytometry. (B) The cell cycle of HT-29 cells was detected by flow cytometry. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* the untreated control. The experiment was repeated at least three times.

known that the ROS/AMPK/mTOR pathway affects the development of tumors and regulates the efficacy of anticancer drugs. Here, according to fluorescence microscopy, *β*-elemene increased the ROS levels in DLD-1 and HT-29 cells in a concentration-dependent manner (Fig. 7A). We further found that the phosphorylation of AMPK was enhanced by *β*-elemene and that the phosphorylation of mTOR was weakened by *β*-elemene in both the cell lines (Fig. 7B). Collectively, these data suggested that *β*-elemene induced autophagy was associated with the activation of ROS/AMPK/mTOR pathway in human CRC cells.

β-Elemene reduces CRC tumor growth in vivo

A mouse-xenograft model inoculated with mouse colon carcinoma HT-29 cells was established to evaluate the antitumor effects of *β*-elemene *in vivo*. 5-FU was used as a positive control. The data showed that *β*-elemene significantly inhibited tumor growth evidenced by the decreased tumor weight and the increased inhibitory rate (Figs. 8A and 8B).

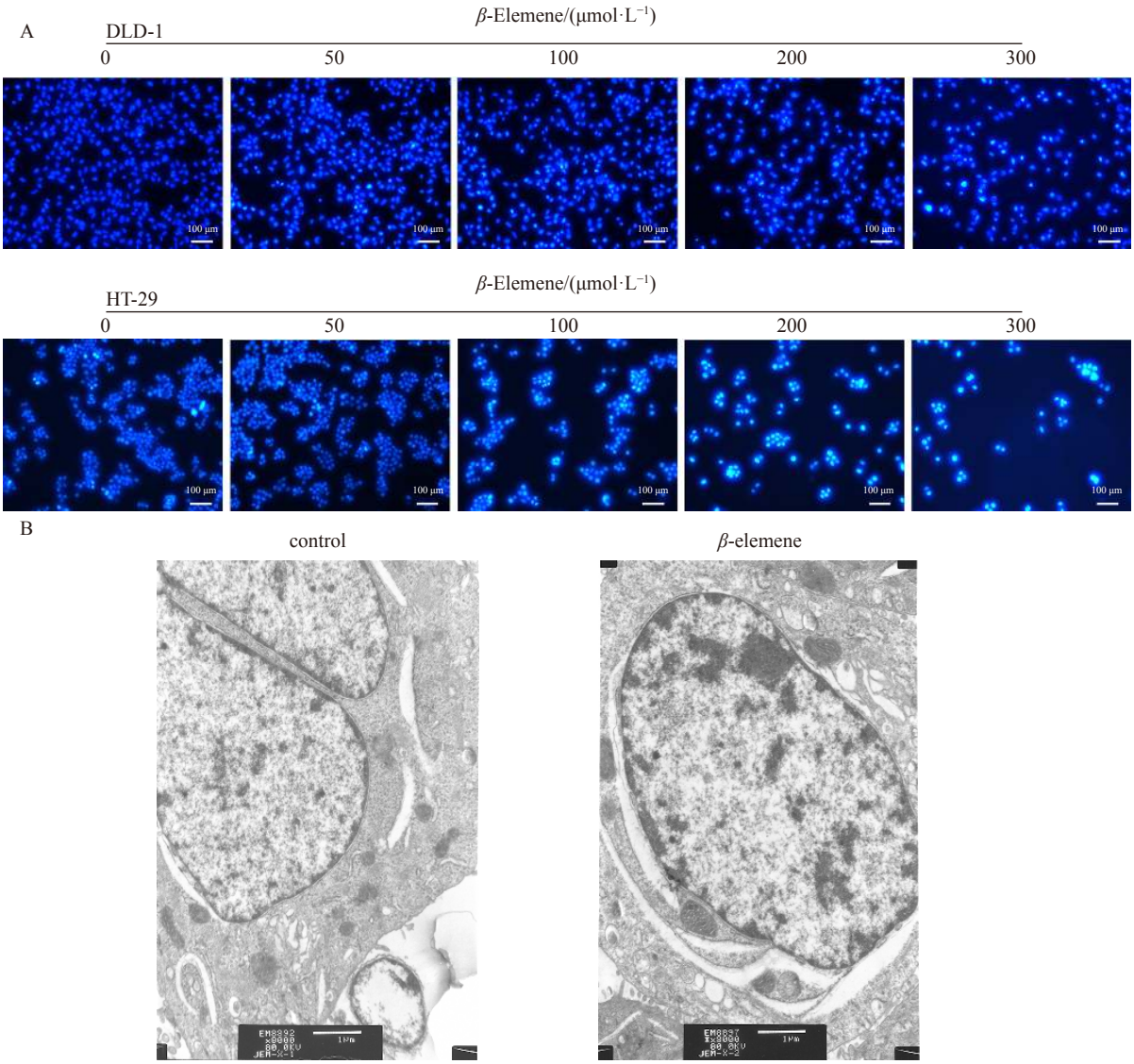

Fig. 3 Effects of *β*-elemene on the nucleus of human CRC cells. (A) The effect of *β*-elemene on cell nucleus was detected by Hoechst 33342 staining. (B) The apoptotic bodies of HT-29 cells were observed by transmission electron microscopy in the presence or absence of *β*-elemene (200 μmol·L^{-1}, 24 h).

H&E staining showed that the tumor cells of the control group were closely arranged, with a large blue-hued nucleus; whereas the tumor cells from *β*-elemene-treated mice were characterized by cytoplasmic condensation, nuclear fragmentation, and cell separation, with necrosis at varying degrees (Fig. 8C). Moreover, the proliferation of these tumor cells was examined by immunohistochemical staining (Ki-67-positive). *β*-Elemene significantly reduced the number of Ki-67 positive cells (Fig. 8D). TUNEL assay was used to detect the apoptotic cells in tumor tissues. The increased staining intensities indicated the degree of DNA damage induced by *β*-elemene (Fig. 8E). *β*-Elemene-induced apoptosis was further confirmed by the increased levels of caspase-3 *in vivo* (Fig. 8F). In addition, *β*-elemene increased the expression of LC3B-II in tumor tissues (Fig. 8G), indicating the accumulation of autophagosome. These results indicated that *β*-elemene exerted anti-CRC effects through inducing apoptosis and autophagy *in vivo*.

Discussion

Cancer is the leading cause of death in China, due to high morbidity and mortality. CRC is commonly diagnosed and identified as one of the leading causes of cancer death. At present, the treatment of CRC is usually performed by surgical resection of the lesion supplemented by medical treatment [16]. But for patients who are clinically intolerable or in the advanced cancer stage, most of the managements eventually fail. Radiotherapy and chemotherapy works to certain extents, but more adverse reactions seriously affect the quality of life of patients [17]. Therefore, there is still a lack of effective and low-toxic anti-tumor drugs for the treatment of CRC.

As a new type of anti-tumor drug, *β*-elemene has been

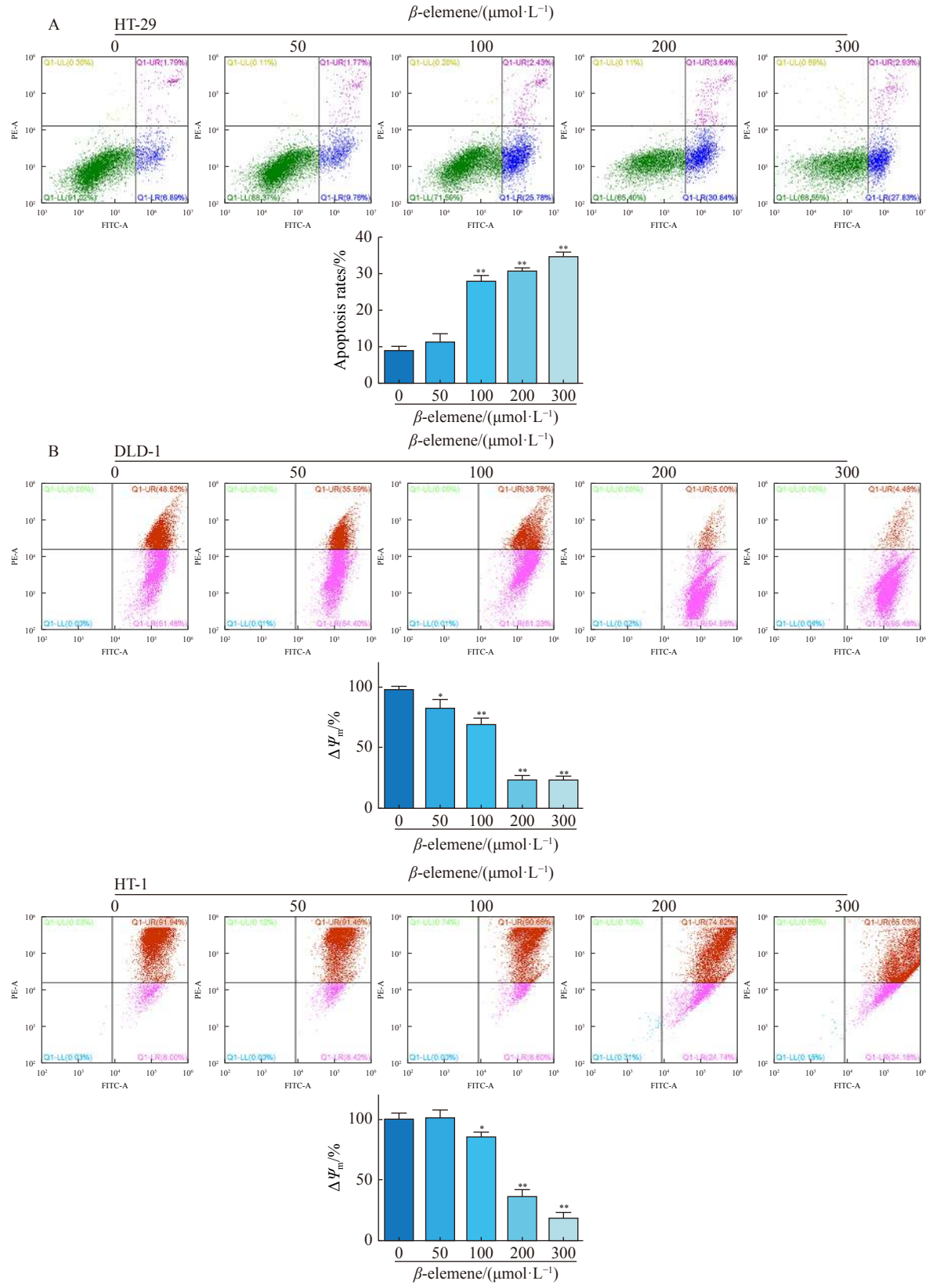

Fig. 4 *β*-Elemene induces the apoptosis of human CRC cells. (A) The effect of *β*-elemene on cell membrane phosphatidylserine valgus in HT-29 cells was detected by Annexin V/PI double staining. (B) The change of cell mitochondrial membrane potential in DLD-1 and HT-29 cells treated with *β*-elemene for 24 h was detected by JC-1 staining. Bars represent means ± SD of three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* non-treated control

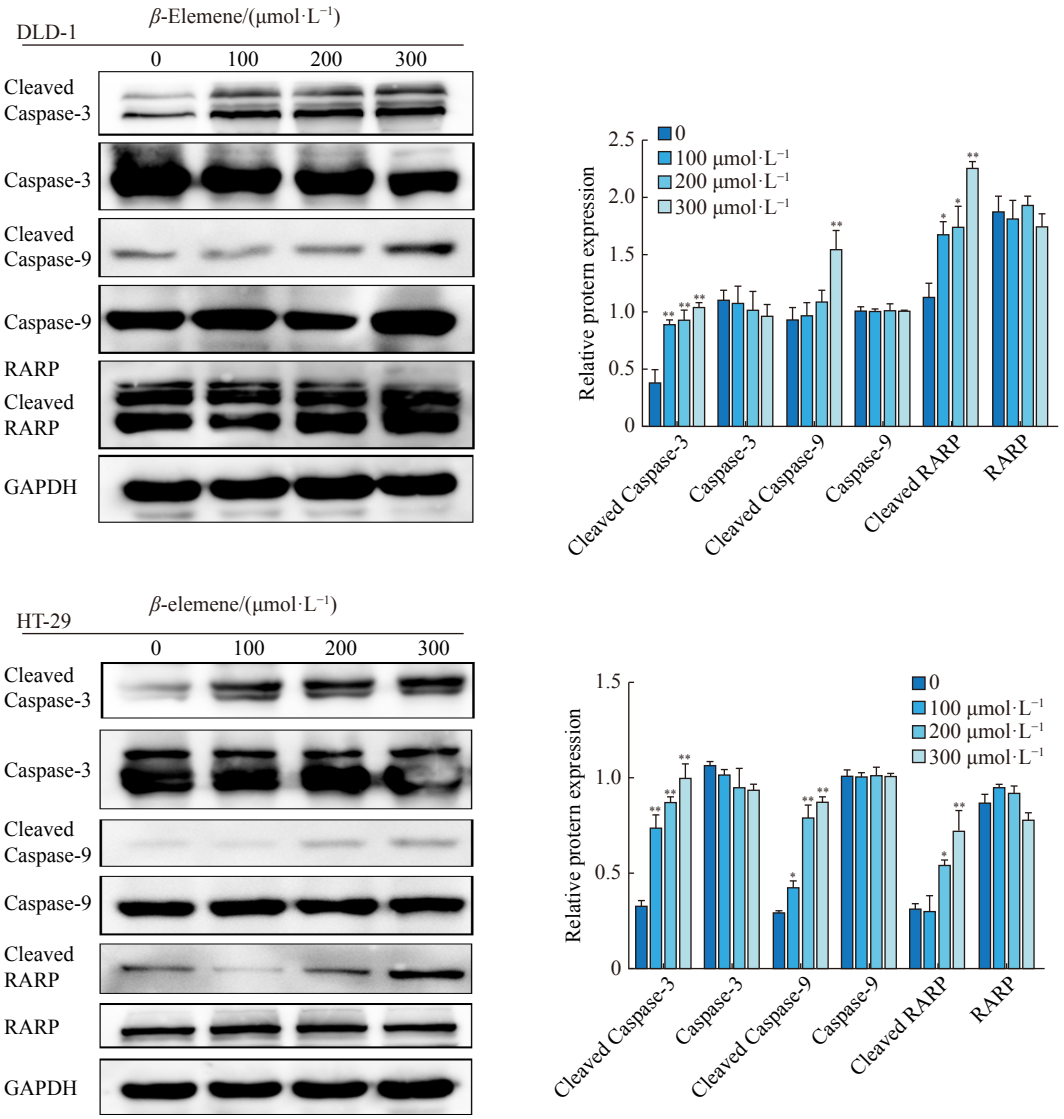

Fig. 5 *β*-Elemene regulates the expression of apoptosis-related proteins in human CRC cells. Western blot analysis of cleaved Caspase-3, Caspase-9 and PARP levels in cells treated with *β*-elemene. GAPDH served as a loading control for all Western blot analyses. Bars represent means ± SD of three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* non-treated control

used as injection, emulsion and other dosage forms for the treatment of cancers such as brain cancer, lung cancer, liver cancer and cancerous ascites. *β*-Elemene exhibits inhibitory effects on tumor proliferation, tumor angiogenesis, invasion and metastasis [18]. Studies have found that *β*-elemene blocked the cell cycle of glioblastoma [19], and affected the expression of apoptosis-related genes such as caspase-3 and survivin in glioma cells and bladder cancer cells [20-21]. *β*-Elemene was also found to regulate epithelial-mesenchymal transition in breast cancer cells [22]. Further studies have shown that *β*-elemene inhibited the proliferation of CRC cells such as CCL-222, CCL-225, and COLO 205 cells [23-24]. Since little research has been conducted to evaluate the effects of *β*-elemene on CRC, and the specific characteristics and mechanism of its effects have not been mentioned, this study was designed to investigate the effects of *β*-elemene on CRC in detail using *in vivo* and *in vitro* experiments and to uncover the possible molecular mechanisms. In the colon cancer HT-29 xenograft model of nude mice, *β*-elemene reduced the weight of tumor xenografts, down-regulated the expression of tumor growth factor Ki-67, decreased the volume of tumor cell mass, and partially caused necrosis in tumor cells. These effects indicated that *β*-elemene inhibited the proliferation of transplanted tumors. The *in vitro* results also showed that *β*-elemene significantly inhibited the viability and proliferation cloning ability of human CRC cells. Cancer cells lose cell cycle control and exhibit the ability to indefinitely proliferate, becoming "immortal" cells. The G_2/M phase checkpoint plays a key role in the cell cycle process and is important for cell growth [25]. Previous studies showed that *β*-elemene can arrest non-small-cell lung cancer cells at the G_2/M phase [26]. Consistently, it was found that *β*-elemene dose-dependently

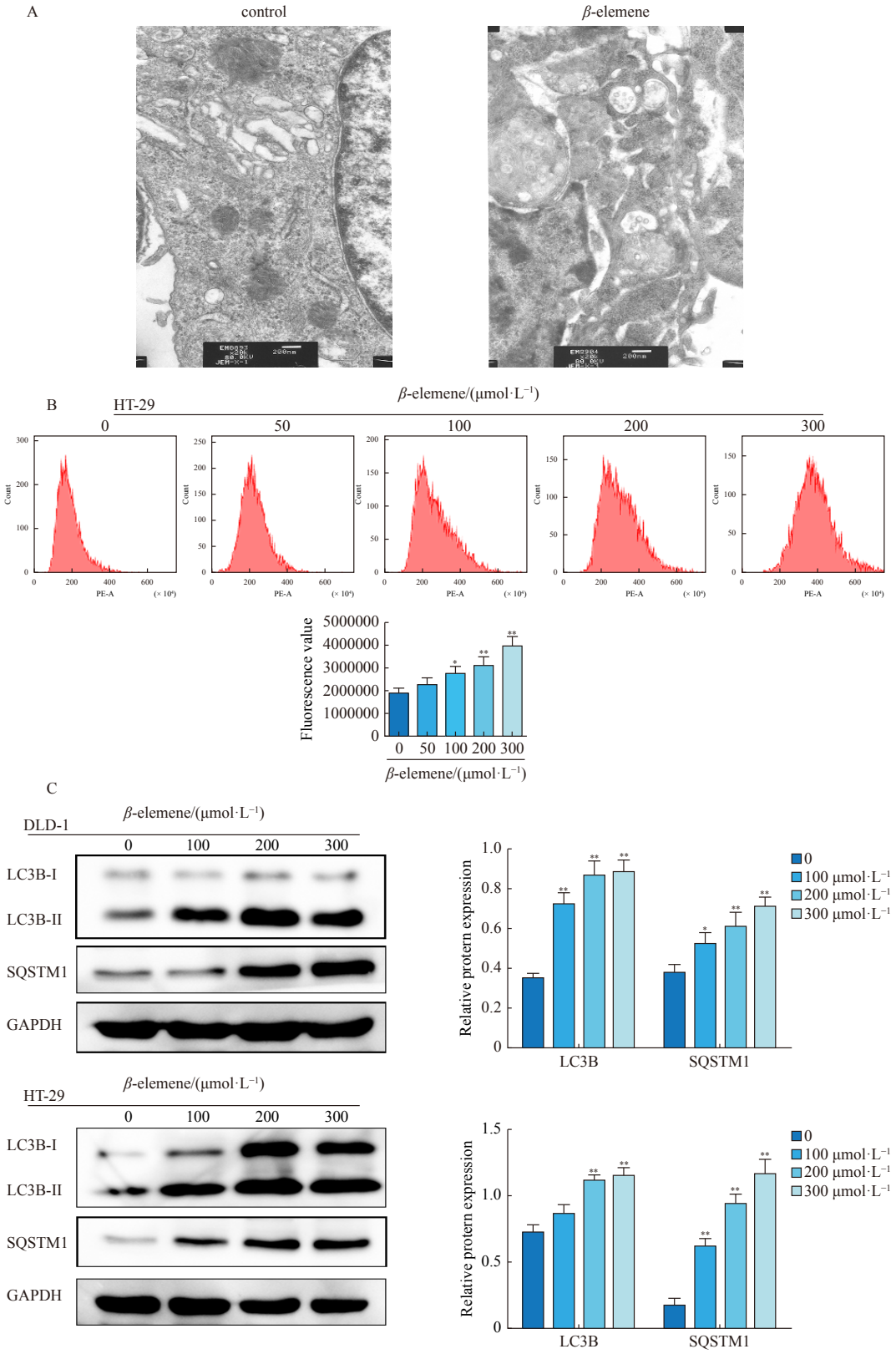

Fig. 6 *β*-Elemene induces the autophagy of human CRC cells. (A) Autophagosomes in HT-29 cells were observed by transmission electron microscopy in the presence or absence of *β*-elemene (300 μmol·L^{-1}, 24 h). (B) The formation of acidic autophagy cells was detected by acridine orange staining. (C) The expression of LC3B and SQSTM1 protein was detected by Western blot. GAPDH served as a loading control for all Western blot analysis. Bars represent means ± SD of three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* non-treated control

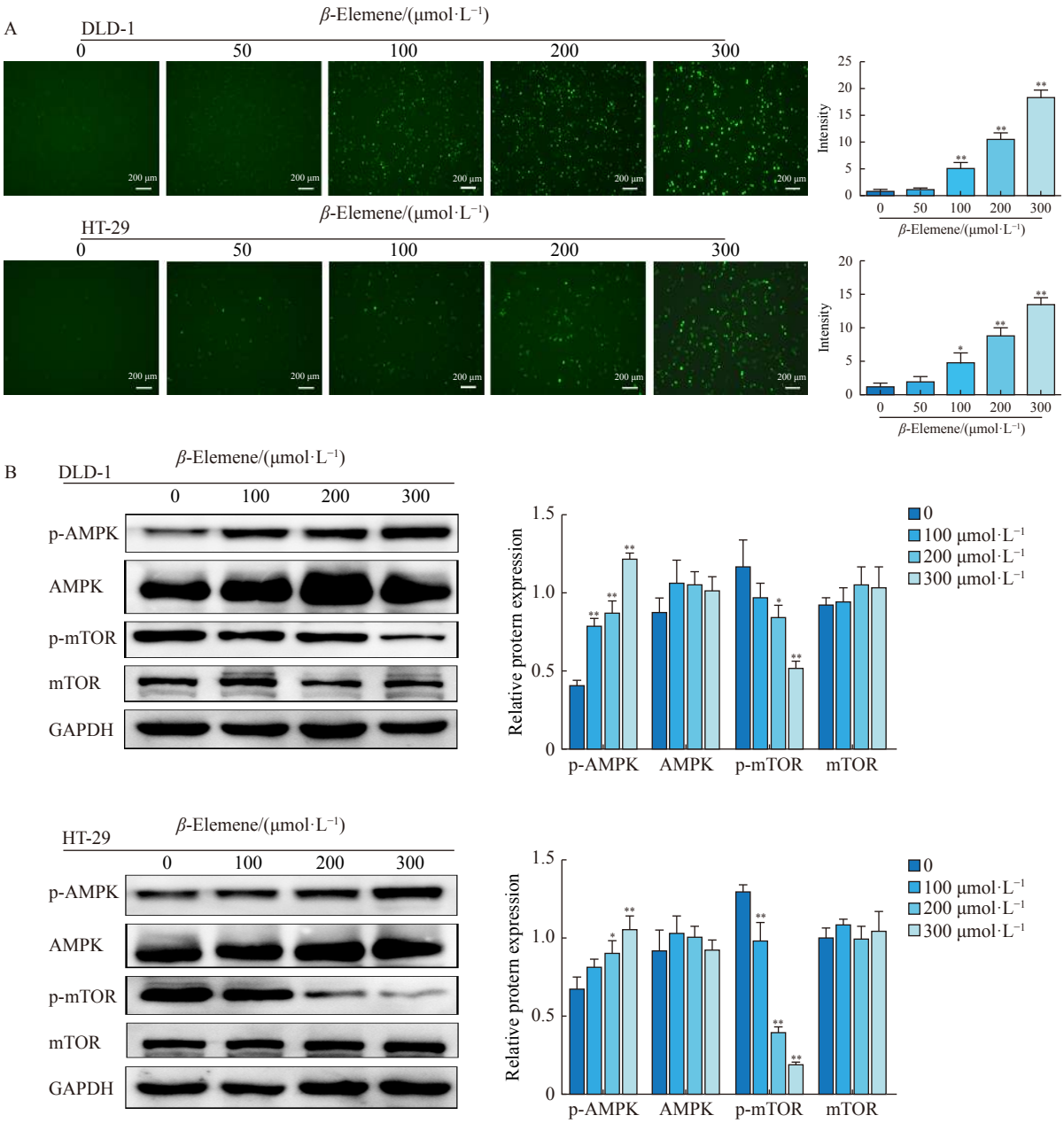

Fig. 7 Effects of *β*-elemene on the ROS /AMPK/mTOR pathway in human CRC cells. (A) The formation of acidic autophagy cells was detected by DCFH-DA staining. (B) Western blot analysis of the phosphorylation of AMPK/mTOR in cells treated with *β*-elemene. GAPDH served as a loading control for all Western blot analysis. Bars represent means ± SD of three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* non-treated control

induced CRC cells at the G_2/M phase in this study, which indicated that the inhibitory effect of *β*-elemene on the proliferation of CRC cells is mediated through inducting cell cycle arrest.

Abnormal regulation of apoptosis is an important marker of cancer, and direct or indirect induction of tumor cell apoptosis is one of the important mechanisms of action of almost all drugs to exert anti-tumor effects [27]. The current study investigated the initial mechanism of *β*-elemene-induced apoptosis in CRC cells. The results showed that the part of the nucleus of *β*-elemene-treated CRC cells was cleaved into pieces, which was accompanied by nuclear chromatin condensation, decreased mitochondrial membrane potential, and occurrence of cell membrane phosphatidylserine. The treated cells exhibited apoptotic bodies, and the levels of cleaved-caspase-3/9/PARP increased after *β*-elemene treatment. The experiments *in vivo* presented the same results. These tumor cell changes suggested that *β*-elemene is an effective active ingredient of traditional Chinese herbal medicine that can induce apoptosis in colorectal cancer.

Autophagy is a decisive factor in the balance between cell death and survival. When the body is subjected to unfa-

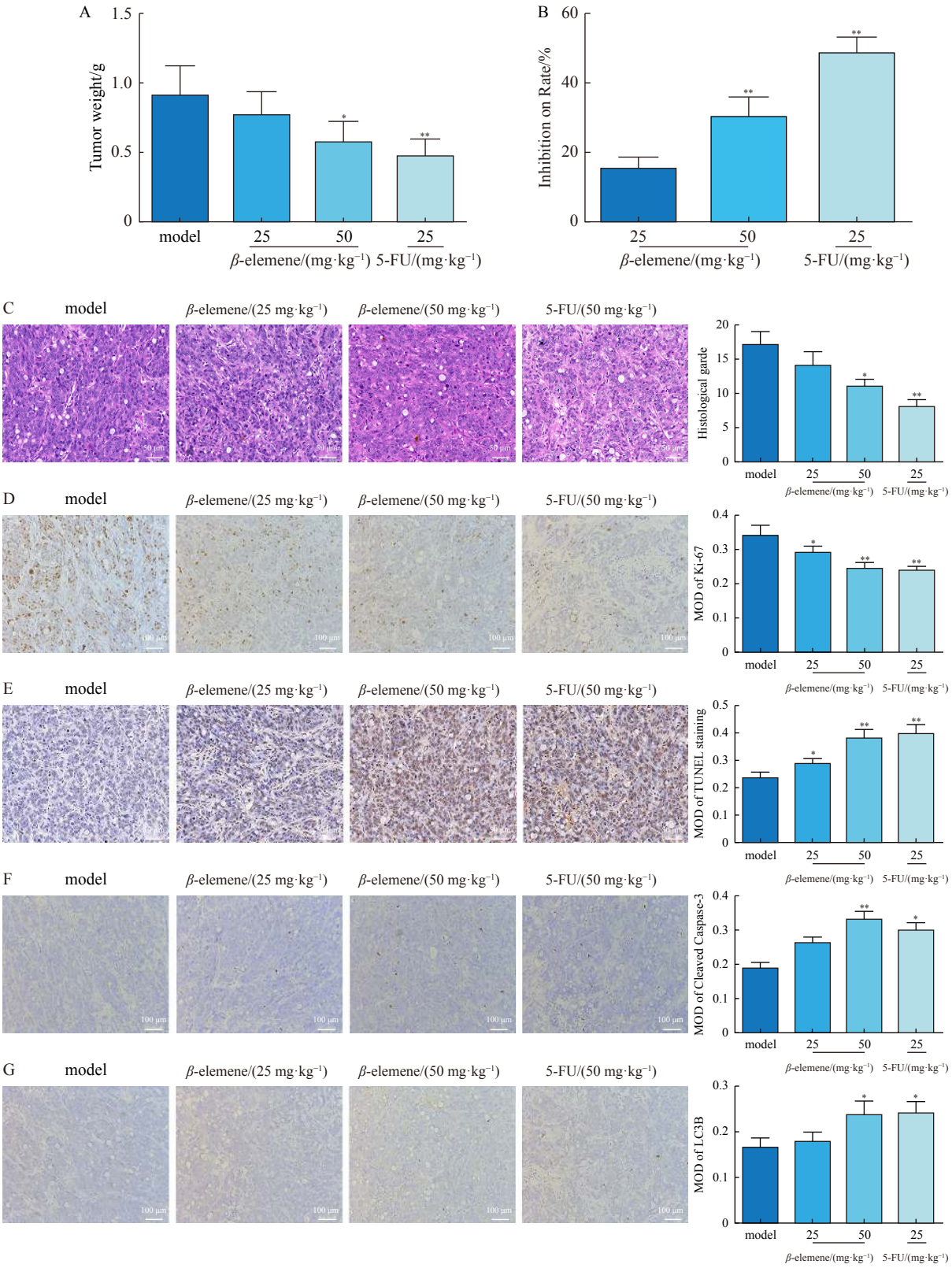

Fig. 8 *β*-Elemene inhibits the growth of colon cancer xenografts *in vivo*. (A) The weight of tumor tissues of treated and control mice. (B) The inhibitory rate of tumor growth of *β*-elemene in nude mice. (C) The difference of transplanted tumor in each group was observed by HE staining. (E) The apoptosis of transplanted tumor was detected by TUNEL staining. (D, F, and G) The expression of Ki-67, cleaved caspase-3 and LC3B were detected by immunohistochemistry. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *vs* the untreated control. The experiment was repeated at least three times.

vorable stress, it causes a programmed death that is different from necrosis and apoptosis, called autophagic cell death [28]. The current study preliminarily explored the role of *β*-elemene in the treatment of CRC *via* autophagy. The results showed that *β*-elemene promoted the increase of intracellular acidic vesicle organelles (autophagy lysosomes), and increased the expression of autophagy markers LC3B and SQSTM1 in human CRC cells, which indicated that *β*-elemene can increase the formation of autophagosomes. The process of autophagy was highly dynamic and multistage. Autophagosome is only an intermediate structure in the entire autophagy pathway. Autophagic flux is a dynamic and continuous concept, covering the entire process of autophagosome formation, autophagy substrate transport to lysosome and degradation in lysosome [29]. Autophagic flux analysis based on autophagic degradation can further illustrate autophagic activity. The results of this study only clarified the formation of autophagosomes, and did not explore the subsequent accumulation and degradation of autophagosomes, which is the weakness of this study. As a stress protein, SQSTM1 selectively removed protein polymers and damaged organelles (such as the mitochondria and peroxisomes) in the cells, while the increase in its level was accompanied by the process of autophagy activation [30]. The up-regulation of SQSTM1 may further suggested the accumulation of autophagosomes and the deficiency of its following degradation. Finally, the activation of autophagic flux was investigated in *β*-elemene-treated colorectal cancer.

The ROS/AMPK/mTOR pathway affects the development of tumors and regulates the role of anti-cancer drugs. Many studies have shown that ROS is not only related to the occurrence and development of tumors, but also closely related to the chemical treatment of tumors. High levels of ROS affect tumor cell apoptosis or autophagy, which plays an important role in the regulation of tumor development [31-32]. AMPK participates in the body's metabolic pathway and regulates the synthesis and decomposition of ATP as an energy balancer in cells. In cancer, AMPK acts as a stress response molecule with dual significance for cancer development and cancer resistance. Targeting AMPK has become a new strategy for prevention and treatment of cancer [33]. mTOR is a major regulator of cell growth control. Cancer cells can use mTOR signaling to drive tumor growth and development [34]. Studies [35-36] found that the ROS/AMPK/mTOR pathway mediated the apoptosis of human ovarian cancer A2780 cells induced by macranthoside B, and regulated HSP60 to affect the proliferation and epithelial-mesenchymal transition of glioblastoma. In our current study, we investigated the role of the ROS/AMPK/mTOR pathway in the treatment of CRC with *β*-elemene. The results showed that *β*-elemene increased ROS levels, enhanced the phosphorylation of AMPK, and reduced the phosphorylation of mTOR in human CRC cells. These data suggested that *β*-elemene regulates apoptosis and autophagy in CRC cells via the ROS/AMPK/mTOR pathway, and exerts anti-CRC effects. The ROS/AMPK/mTOR cascade is closely related to apoptosis and autophagy, and its molecular mechanism is complex [33, 37-38]. Therefore, further studies should be performed to explore the characteristics and mechanisms of the ROS/AMPK/mTOR pathway involved in apoptosis and autophagy in *β*-elemene-treated CRC cells.

In summary, our study demonstrated that *β*-elemene effectively inhibited the proliferation of human CRC cells through arresting at the S and G_2/M phase, stimulating apoptosis and mediating autophagosome accumulation *in vitro* and *in vivo*. The ROS/AMPK/mTOR signaling contributed to these effects. These findings provided novel molecular basis for developing *β*-elemene as a novel anti-cancer lead for the treatment of CRC.

References

[1] Sekiguchi M, Matsuda T, Saito Y. Surveillance after endoscopic and surgical resection of colorectal cancer [J]. *Best Pract Res Clin Gastroenterol*, 2016, **30**(6): 959-970.

[2] Chen W, Zheng R, Baade PD, *et al*. Cancer statistics in China, 2015 [J]. *CA Cancer J Clin*, 2016, **66**(2): 115-132.

[3] Diagnosis ATGF. Chinese Society of Clinical Oncology (CSCO) diagnosis and treatment guidelines for colorectal cancer 2018 (English version) [J]. *Chin J Cancer Res*, 2019, **31**(1): 117-134.

[4] Siegel RL, Miller KD, Goding SA, *et al*. Colorectal cancer statistics, 2020 [J]. *CA Cancer J Clin*, 2020, **70**(3): 145-164.

[5] Grilo AL, Mantalaris A. Apoptosis: A mammalian cell bioprocessing perspective [J]. *Biotechnol Adv*, 2019, **37**(3): 459-475.

[6] Rybstein MD, Bravo-San PJ, Kroemer G, *et al*. The autophagic network and cancer [J]. *Nat Cell Biol*, 2018, **20**(3): 243-251.

[7] Linder B, Kogel D. Autophagy in cancer cell death [J]. *Biology (Basel)*, 2019, **8**(4): 82.

[8] Su Z, Yang Z, Xu Y, *et al*. Apoptosis, autophagy, necroptosis, and cancer metastasis [J]. *Mol Cancer*, 2015, **14**: 48.

[9] Mowers EE, Sharifi MN, Macleod KF. Functions of autophagy in the tumor microenvironment and cancer metastasis [J]. *FEBS J*, 2018, **285**(10): 1751-1766.

[10] Bi YH, Zhang LH, Chen SJ, *et al*. Antitumor mechanisms of Curcumae Rhizoma based on network pharmacology [J]. *Evid-Based Compl Alt*, 2018, **2018**: 4509892.

[11] Li W, Hong B, Li Z, *et al*. GC-MS method for determination and pharmacokinetic study of seven volatile constituents in rat plasma after oral administration of the essential oil of Rhizoma Curcumae [J]. *J Pharm Biomed Anal*, 2018, **149**: 577-585.

[12] Deng MM, Zhang Y, Liu BF, *et al*. *β*-Elemene inhibits peritoneal metastasis of gastric cancer cells by modulating FAK/Claudin-1 signaling [J]. *Phytother Res*, 2019, **33**(9): 2448-2456.

[13] Zhai BT, Zhang NN, Han XM, *et al*. Molecular targets of *β*-elemene, a herbal extract used in traditional Chinese medicine, and its potential role in cancer therapy: A review [J]. *Biomed Pharmacother*, 2019, **114**: 108812.

[14] Ooko E, Kadioglu O, Greten HJ, *et al*. Pharmacogenomic characterization and isobologram analysis of the combination of ascorbic acid and curcumin-Two main metabolites of *Curcuma longa*-in cancer cells [J]. *Front Pharmacol*, 2017, **8**: 38.

[15] Zhai BT, Zeng YY, Zeng ZW, *et al*. Drug delivery systems for elemene, its main active ingredient *β*-elemene, and its derivatives in cancer therapy [J]. *Int J Nanomedicine*, 2018, **13**: 6279-6296.

[16] Brown KGM, Solomon MJ, Mahon K, *et al*. Management of colorectal cancer [J]. *Postgrad Med J*, 2019, **366**: l4561.

[17] Vermeer NC, Snijders HS, Holman FA, *et al*. Colorectal cancer screening: Systematic review of screen-related morbidity and mortality [J]. *Cancer Treat Rev*, 2017, **54**: 87-98.
[18] Jiang SY, Ling CH, Li W, *et al*. Molecular mechanisms of anticancer activities of *β*-elemene: Targeting hallmarks of cancer [J]. *Anticancer Agents Med Chem*, 2016, **16**(11): 1426-1434.
[19] Zhu TZ, Xu YH, Dong B, *et al*. *β*-Elemene inhibits proliferation of human glioblastoma cells through the activation of glia maturation factor β and induces sensitization to cisplatin [J]. *Oncol Rep*, 2011, **26**(2): 405-413.
[20] Zhang H, Xu F, Xie T, *et al*. *β*-Elemene induces glioma cell apoptosis by downregulating survivin and its interaction with hepatitis B X-interacting protein [J]. *Oncol Rep*, 2012, **28**(6): 2083-2090.
[21] Chen X, Wang Y, Luo HM, *et al*. *β*-Elemene acts as an antitumor factor and downregulates the expression of survivin, Bcl-xL and Mta-1 [J]. *Mol Med Rep*, 2012, **6**(5): 989-895.
[22] Zhang X, Li YH, Zhang Y, *et al*. Beta-elemene blocks epithelial-mesenchymal transition in human breast cancer cell line MCF-7 through Smad3-mediated down-regulation of nuclear transcription factors [J]. *PLOS ONE*, 2013, **8**(3): e58719.
[23] Li QQ, Wang G, Liang H, *et al*. *β*-Elemene promotes cisplatin-induced cell death in human bladder cancer and other carcinomas [J]. *Anticancer Res*, 2013, **33**(4): 1421-1428.
[24] Li QQ, Wang G, Huang F, *et al*. Antineoplastic effect of *β*-elemene on prostate cancer cells and other types of solid tumour cells [J]. *J Pharm Pharmacol*, 2010, **62**(8): 1018-1027.
[25] Henderson L, Bortone DS, Lim C, *et al*. Classic "broken cell" techniques and newer live cell methods for cell cycle assessment [J]. *Am J Physiol Cell Physiol*, 2013, **304**(10): C927-C938.
[26] Wang G, Li X, Huang F, *et al*. Antitumor effect of *β*-elemene in non-small-cell lung cancer cells is mediated *via* induction of cell cycle arrest and apoptotic cell death [J]. *Cell Mol Life Sci*, 2005, **62**(7-8): 881-93.
[27] Hassan M, Watari H, AbuAlmaaty A, *et al*. Apoptosis and molecular targeting therapy in cancer [J]. *Biomed Res Int*, 2014, **2014**: 150845.
[28] Janku F, McConkey DJ, Hong DS, *et al*. Autophagy as a target for anticancer therapy [J]. *Nat Rev Clin Oncol*, 2011, **8**(9): 528-539.
[29] Neufeld TP. Autophagy and cell growth--the yin and yang of nutrient responses [J]. *J Cell Sci*, 2012, **125**(Pt 10): 2359-2368.
[30] Katsuragi Y, Ichimura Y, Komatsu M. p62/SQSTM1 functions as a signaling hub and an autophagy adaptor [J]. *FEBS J*, 2015, **282**(24): 4672-4678.
[31] Sodrul I, Wang C, Chen X, *et al*. Role of ginsenosides in reactive oxygen species-mediated anticancer therapy [J]. *Oncotarget*, 2018, **9**(2): 2931-2950.
[32] Tsai CY, Chen CY, Chiou YH, *et al*. Epigallocatechin-3-gallate suppresses human herpesvirus 8 replication and induces ROS leading to apoptosis and autophagy in primary effusion lymphoma cells [J]. *Int J Mol Sci*, 2017, **19**(1): 16.
[33] Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression [J]. *Biochim Biophys Acta*, 2013, **1833**(12): 3481-3498.
[34] Ardestani A, Lupse B, Kido Y, *et al*. mTORC1 signaling: A double-edged sword in diabetic β cells [J]. *Cell Metab*, 2018, **27**(2): 314-331.
[35] Tang HP, Li J, Liu XH, *et al*. Down-regulation of HSP60 suppresses the proliferation of glioblastoma cells *via* the ROS/AMPK/mTOR pathway [J]. *Sci Rep*, 2016, **6**: 28388.
[36] Shan Y, Guan FQ, Zhao XZ, *et al*. Macranthoside B induces apoptosis and autophagy *via* reactive oxygen species accumulation in human ovarian cancer A2780 cells [J]. *Nutr Cancer*, 2016, **68**(2): 280-289.
[37] Rad E, Murray JT, Tee AR. Oncogenic signalling through mechanistic target of rapamycin (mTOR): A driver of metabolic transformation and cancer progression [J]. *Cancers (Basel)*, 2018, **10**(1): 5.
[38] Paquette M, El-Houjeiri L, Pause A. mTOR pathways in cancer and autophagy [J]. *Cancers (Basel)*, 2018, **10**(1): 18.

Cite this article as: WANG Guo-Yu, ZHANG Lei, GENG Ya-Di, WANG Bin, FENG Xiao-Jun, CHEN Zhao-Lin, WEI Wei, JIANG Ling. *β*-Elemene induces apoptosis and autophagy in colorectal cancer cells through regulating the ROS/AMPK/mTOR pathway [J]. *Chin J Nat Med*, 2022, **20**(1): 9-21.