Abstract. Previous studies by our group have demonstrated that extract of clove exhibits potent anticancer effects in vitro and in vivo. In the present study, the effect of an extracted and isolated active fraction of clove (AFC) on induction of cellular apoptosis in human colorectal cancer HCT-116 cells was investigated by morphological observation, flow cytometry, and western blotting analysis. The results revealed that AFC induced apoptosis of HCT-116 cells. AFC also induced autophagy, demonstrated by increased punctuate microtubule-associated protein 1A/1B-light chain 3 (LC3) staining, and LC3-II and Beclin-1 protein expression levels. Furthermore, the autophagy inhibitors 3-MA and baflomycin A1 potentiated the pro-apoptotic activity of AFC in HCT-116 cells. AFC also inhibited the phosphorylation of the phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin signaling pathway. The present study may improve the existing understanding of the anticancer mechanisms of clove and provide a scientific rationale for AFC to be further developed as a promising novel anticancer agent for the treatment of colorectal cancer.

Introduction

Colorectal cancer (CRC) is among the most common types of cancer and the third leading cause of cancer-associated mortality worldwide. Therefore, it is an important topic among oncologists and cancer biologists (1-3). Although the mortality rate of CRC has decreased significantly with early detection and surgery and chemotherapy treatments, recurrence and drug resistance have become more common (4). Therefore, there is an urgent requirement to develop novel anticancer drugs and/or therapeutic strategies for the treatment of colorectal cancer.

Clove, the dried bud of Syzygium aromaticum, is a traditional medicinal herb widely used in Asian countries. Clove has been indicated to possess various biological properties including anti-inflammatory (5,6), antiviral (7), antibacterial (8), antioxidant (8,9) and antitumor (8,10,11) activities. Our previous study demonstrated that the active fraction of clove (AFC) was effective against various types of cancer cell, including lung, breast, liver, pancreatic, ovarian and cervical cancer (12). Two components of AFC have been identified as demonstrating cytotoxicity against various types of cancer cells: Oleanonic acid (OA) and eugenol (12). It was also demonstrated that OA was able to induce apoptosis of cancer cells via the mitochondrial pathway (13). Combination of OA and fluorouracil (5-FU) treatments synergistically potentiated the cytotoxicity of 5-FU against human pancreatic cancer Pan-28 cells (13). AFC was more effective than a single isolated component of OA or eugenol against human colon cancer HT-29 xenografts in vivo (12). However, the mechanistic action of AFC remains unclear. Therefore, the present study had the interesting aim of elucidating the mechanism of action of AFC.

Apoptosis (programmed cell death) is a highly regulated and controlled process and serves a crucial role in the development and treatment of cancer (14-16). Apoptosis occurs through two major molecular pathways, the intrinsic (mitochondrial) and the extrinsic (death receptor-mediated) pathways (16,17). The intrinsic pathway involves the mitochondria and mainly affects the Bcl-2 and caspase families (17). The extrinsic pathways involves death signals, including TNF-α with TNF receptor 1 (TNFR1) and activates caspase-8 to cleave procaspase-3 into its active form (16). Apoptosis is an important anticancer mechanism and numerous anticancer drugs execute their anticancer activity via induction of apoptosis (18).

Autophagy is a survival-promoting pathway and serves a complicated role in cell development, growth and tumorigenesis to regulate inhibition of cancer cell proliferation or promotion of cancer cell survival (19-22). Numerous anticancer
agents display antitumor activity via autophagy of cancer cells (23-25). The phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin signaling pathway is a major signal transduction cascade involved in cell proliferation, survival and metabolism, and it serves an important role in the development and therapy of colorectal cancer (26,27). Upregulation of PI3K expression results in the inhibition of apoptosis in colon cancer SW480 cells (28). The PI3K/Akt/mTOR signaling pathway is also an important therapeutic target and a previous study demonstrated that the dual PI3K/mTOR inhibitor, NVP-BEZ235, was effective against colorectal cancer cells in vitro and in vivo (29). The PI3K/Akt/mTOR signaling pathway is also involved in the autophagic process, and activation of the pathway attenuates autophagy in cancer cells. A recent study also demonstrated that OA, one of the main components of AFC, is capable of inducing protective autophagy in cancer cells via the PI3K/Akt/mTOR signaling pathway (30). However, the effect of AFC on autophagy remains unclear. In the present study, AFC-induced autophagic effects were evaluated in human colorectal cancer HCT-116 cells by morphological observation, flow cytometry and western blotting. The results revealed that AFC induced apoptosis via the PI3K/Akt/mTOR-mediated autophagic pathway in colorectal cancer HCT-116 cells.

Materials and methods

Reagents and antibodies. Rapamycin and 3-methyladenine (3-MA) were purchased from MedChem Express Co., Ltd. (Shanghai, China). Bafilomycin A1 (BA) was purchased from Beijing Huaxin MEIKO Biotechnology Co., Ltd. (Beijing, China). The Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Diego, CA, USA). Cell culture media, DMEM, RPMI-1640, and McCoy’s 5A were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cell Counting Kit-8 (CCK-8), Hoechst 33342, Ad-mCherry-GFP-LC3B, dimethyl sulfoxide (DMSO), fetal bovine serum (FBS), penicillin, streptomycin, monoclonal antibodies of β-actin (cat. no. AA128-1), horseradish peroxidase (HRP)-labeled goat anti-rat IgG(H+L) (#A0216) and HRP-labeled goat anti-rabbit IgG(H+L) (cat. no. A0208) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). LY294002, the PI3K inhibitor, was dissolved in DMSO and diluted with culture medium to final concentrations of 25, 50, 100, 200 or 400 µg/ml for the treatment of HCT-116 cells for 24, 48 or 72 h. The PI3K inhibitor, LY294002, and the autophagy-lysosomal inhibitor, BA, were dissolved in DMSO and diluted with culture medium. The final concentration of DMSO in the test solutions was <0.1%. This concentration of DMSO did not cause any adverse responses in cells. 3-MA was dissolved in heated sterile double distilled water to achieve a 100 mM stock solution, and was then diluted with culture medium for a final concentration of 2 mM. IGF-I (a PI3K activator) was reconstituted to 10 µg/ml in sterile PBS, and diluted with culture medium for a final concentration of 50 ng/ml. HCT-116 cells were pretreated with 10 µM LY294002, 1 nM BA, 2 mM 3-MA or 50 ng/ml IGF-I for 1 h, then further treated with 100 µg/ml AFC for 48 h. Cells were treated with fresh medium without serum as a vehicle control.

Drug preparation and treatment. AFC was diluted with cell culture medium to final concentrations of 25, 50, 100, 200 and 400 µg/ml for the treatment of HCT-116 cells for 24, 48 or 72 h. The PI3K inhibitor, LY294002, and the autophagy-lysosomal inhibitor, BA, were dissolved in DMSO and diluted with culture medium. The final concentration of DMSO in the test solutions was <0.1%. This concentration of DMSO did not cause any adverse responses in cells. 3-MA was dissolved in heated sterile double distilled water to achieve a 100 mM stock solution, and was then diluted with culture medium for a final concentration of 2 mM. IGF-I (a PI3K activator) was reconstituted to 10 µg/ml in sterile PBS, and diluted with culture medium for a final concentration of 50 ng/ml. HCT-116 cells were pretreated with 10 µM LY294002, 1 nM BA, 2 mM 3-MA or 50 ng/ml IGF-I for 1 h, then further treated with 100 µg/ml AFC for 48 h. Cells were treated with fresh medium without serum as a vehicle control.

Cell viability assay. Cell viability was determined by CCK-8 assay as previously described (12). Briefly, cells at 80-90% confluency were seeded in 96-well plates at a density of 5x10³ cells/well. After a 24-h incubation 25, 50, 100, 200 or 400 µg/ml AFC, or vehicle control, was added to the wells. After 24, 48 or 72 h 10% CCK-8 reagent (10 µl/well) was added and incubated for 1 h at 37°C. The absorbance value (optical density; OD) was measured at a wavelength of 450 nm with a SpectraMAX M3 microplate spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA, USA) and the cell viability ratio was calculated using the following formula: Cell viability rate = (OD of the experimental group/OD of the control group) x100%. The half maximal inhibitory concentration (IC₅₀) values were determined using a nonlinear best fit method.
by GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). All experiments were performed 3 times in triplicate.

**GFP-LC3 transfection.** HCT-116 cells were seeded onto coverslips in a 24-well plate at a density of 5x10^4 cells/well and were transfected with Ad-mCherry-GFP-LC3B. Following 24 h of culture, the cells were treated with AFC (100 µg/ml) or medium (control) for 48 h then fixed with 4% polyoxy-methylene and observed with an EVOS™ FL Imaging system (AMF4300; Thermo Fisher Scientific Inc.). Autophagic cells presenting ≥5 mRFP-GFP-LC3 dots were counted. All experiments were performed 3 times in triplicate.

**Apoptotic assay of HCT-116 cells by Hoechst 33258 staining.** Morphological assessment of apoptotic cells was analyzed using Hoechst 33258 staining as previously described (31). Briefly, HCT-116 cells were seeded in a 24-well plate at a density of 5x10^4 cells/well. Following culture for 24 h, the cells were treated with 25, 50 or 100 µg/ml AFC or the same volume of vehicle control. Following incubation for another 24 h, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and the medium was removed and washed with PBS for 15 min. The cells were treated with 10 µg/ml Hoechst 33258 staining at room temperature for 10 min, and washed with PBS for 15 min in the dark to reduce background. The morphology of the cells was visualized and photographed under a DMR fluorescence microscope at x400 magnification (Leica Microsystems GmbH, Wetzlar, Germany) using fluorescence excitation at 340 nm. The apoptotic index was calculated using the following formula: Apoptotic index = apoptotic cell number/total cell number x 100%. A minimum of 4 fields of view of each well, containing ≥500 cells, were required to calculate the rate of apoptosis. All experiments were performed 3 times in triplicate.

**Observation of autophagosomes by transmission electron microscopy.** HCT-116 cells were seeded in a 24-well plate at a density of 5x10^4 cells/well. Following 24 h of culture, the cells were treated with 25, 50 and 100 µg/ml AFC or the equivalent volume of medium (vehicle control) for 48 h. The cells were collected by trypsinization and washed twice with PBS, then fixed in 2.5% glutaraldehyde for 90 min and post-fixed for 10 min at room temperature, and the medium was removed and washed with PBS for 15 min. The cells were treated with 10 µg/ml Hoechst 33258 staining at room temperature for 10 min, and washed with PBS for 15 min in the dark to reduce background. The morphology of the cells was visualized and photographed under a DMR fluorescence microscope at x400 magnification (Leica Microsystems GmbH, Wetzlar, Germany) using fluorescence excitation at 340 nm. The apoptotic index was calculated using the following formula: Apoptotic index = apoptotic cell number/total cell number x 100%. A minimum of 4 fields of view of each well, containing ≥500 cells, were required to calculate the rate of apoptosis. All experiments were performed 3 times in triplicate.

**Statistical analysis.** All experiments were repeated ≥3 times. Data were analyzed using SPSS 19.0 statistical software (IBM Corporation, Armonk, NY, USA) and expressed as the mean ± standard deviation. One-way analysis of variance and Tukey’s test were used to compare the means among groups. P<0.05 was considered to indicate a statistically significant difference, and highly significant differences were indicated by P<0.01 and P<0.001.
Results

AFC inhibits the proliferation of colorectal cancer cells. We initially investigated the effects of AFC on various types of human colorectal cancer cells using a CCK-8 assay. The results demonstrated that AFC was able to significantly inhibit proliferation of HCT116, HT-29, SW620, HCT8 and LoVo cells (Fig. 1; Table I). Compared with other cells, HCT116 cells were the most sensitive to AFC. Therefore, HCT116 cells were selected for use in subsequent experiments to investigate the potential mechanisms of the effect AFC in human colorectal cancer cells.

AFC inhibits cell viability and induces apoptosis of HCT-116 cells. The effects of AFC on cell viability of human colorectal cancer HCT-116 cells was investigated by CCK-8 assay. The results demonstrated that AFC inhibited the viability of HCT-116 cells in a dose- and time-dependent manner (Fig. 2A and B). Next, the effect of AFC on the induction of apoptosis of HCT-116 cells was investigated by Hoechst 33258 staining, which is commonly used to detect cell apoptosis by observation of chromatin condensation under a fluorescence microscope (31). As demonstrated in Fig. 2C, following treatment with AFC (25, 50 and 100 µg/ml) for 48 h, the cells exhibited typical apoptotic morphological features, including chromatin condensation, nuclear shrinkage and the formation of apoptotic bodies. Annexin V is a sensitive method for detection of early apoptosis of cancer cells by using fluorescein (FITC) as fluorescent probe (32). Therefore, the rate of apoptosis of HCT-116 cells was investigated by flow cytometric analysis using Annexin V-FITC/PI double staining. The results demonstrated that the percentage of apoptotic cells increased when HCT-116 cells were treated with AFC compared with control (Fig. 2D and E). To study the underlying mechanism of AFC-induced apoptosis, the cleavage of PARP, caspase-3, and caspase-9 was analyzed by western blotting. As demonstrated in Fig. 2F and G, AFC cleaved PARP, pro-caspase-3 and pro-caspase-9 into their active forms. These data indicate that AFC inhibits cell viability through inducing apoptosis of HCT-116 cells.

AFC induces autophagy in HCT-116 colorectal cancer cells. To study AFC-induced autophagy, the autophagic flow of LC3-puncta was firstly determined. HCT-116 cells were transfected with Ad-mCherry-GFP-LC3B and GFP-LC3 puncta were observed under a fluorescence microscope. As indicated by Fig. 3A, fluorescence of GFP-LC3 puncta was frequently observed in HCT-116 cells treated with AFC, whereas the cells treated with vehicle control exhibited a relatively homogeneous LC3 expression pattern. In addition, a large number of autophagic bodies and autophagy-lysosomes were observed in HCT-116 cells treated with AFC (Fig. 3B) by using transmission electron microscopy.

It has been well established that the microtubule-associated protein 1A/1B-light chain 3 (LC3) is a central protein in the autophagy pathway and closely associated with autophagosome appearance. Thus, it serves as a reliable marker to monitor autophagy (33). Beclin-1 was the first gene identified to induce autophagy (34). Therefore, the effect of AFC on the expression of LC3 and Beclin-1 was next investigated. As demonstrated by Fig. 3C-E, the expression levels of LC3-II and Beclin-1 in HCT-116 cells were significantly increased by AFC in a dose- and time-dependent manner. These data indicated that AFC treatment not only results in apoptosis, but also induces autophagy in HCT-116 cells.

Inhibition of autophagy enhances AFC-induced apoptosis in HCT-116 cells. It has been documented that autophagy could facilitate cell survival in adverse microenvironments and that inhibition of autophagy leads to increased cytotoxicity and induction of apoptosis (35). The effect of inhibition of autophagy on apoptosis induced by AFC in HCT-116 cells was investigated using 2 inhibitors, 3-MA and BA. 3-MA suppresses class III phosphatidylinositol 3-kinase (PI3K), essential for the initiation of the early stages of autophagy (36), while BA, an inhibitor of the vacuolar-type ATPase, inhibits the fusion of autophagosomes with lysosomes, preventing autophagic degradation (37). As demonstrated in Fig. 4A, cell viability was significantly decreased in HCT-116 cells treated with the combination of AFC (100 µg/ml) and 3-MA (2 mM) or BA (1 nM). The resulting cell viability was 40.3±2.52 and 54.3±4.04%, respectively, compared with 73.2±5.13% for cells treated with AFC alone. This result indicated that the inhibitory effect of AFC on HCT-116 cell proliferation was enhanced by autophagic inhibitors of 3-MA and BA.

Recent studies suggest that increased LC3 expression could reflect either increased autophagosome formation, due to the upregulation of the autophagy-related genes, or the inhibition of autophagy. Therefore, it is crucial to investigate the underlying mechanisms of the effect AFC in human colorectal cancer cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor type</th>
<th>IC50 µg/ml (mean ± standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>Human colorectal cancer</td>
<td>113.5±7.83</td>
</tr>
<tr>
<td>SW620</td>
<td>Human colorectal cancer</td>
<td>174.9±9.52</td>
</tr>
<tr>
<td>HCT8</td>
<td>Human colorectal cancer</td>
<td>211.7±7.29</td>
</tr>
<tr>
<td>HT29</td>
<td>Human colorectal cancer</td>
<td>232.3±15.42</td>
</tr>
<tr>
<td>LoVo</td>
<td>Human colorectal cancer</td>
<td>280.9±12.61</td>
</tr>
</tbody>
</table>

IC50, half maximal inhibitory concentration.
to increased autophagic activity, or reduced autophagosome turnover (33). Therefore, the effects of AFC on LC3 expression in the presence of 3-MA or BA were also studied. As demonstrated in Fig. 4B, 2 mM 3-MA decreased the expression of LC3, but 1 nM BA increased the level of LC3 expression. The opposite effects of 3-MA and BA on LC3 are associated with blocking autophagy at different stages: 3-MA inhibits autophagosome formation, whereas BA prevents degradation of LC3 in autophagolysosomes and in turn increases the LC3 expression level (38). Additionally, 3-MA significantly decreased the LC3 expression induced by AFC, whereas BA increased the LC3 expression induced by AFC treatment (Fig. 4B). These data
LIU et al: ACTIVE FRACTION OF CLOVE INDUCES APOPTOSIS AND AUTOPHAGY

Figure 3. AFC induces autophagy in HCT-116 cells. (A) Stable expression of GFP-LC3 was observed under a fluorescence microscope in HCT-116 cells following treatment with 100 µg/ml AFC for 48 h. Arrows indicate autophagic cells. (B) Autophagy body (thin arrows) and autophagy-lysosome (thick arrows) were observed in HCT-116 cells under a transmission electron microscope (x400, magnification), following treatment with 100 µg/ml AFC for 48 h. (C) Expression of LC3 and Beclin-1 in HCT-116 cells. (D) Quantification of the LC3-II/I protein expression ratio, using actin as a loading control. (E) Quantification of Beclin-1 protein expression, using actin as a loading control. All data are expressed as the mean ± standard deviation of 3 independent experiments in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle control. AFC, active fraction of clove; GFP, green fluorescent protein; LC3, microtubule-associated proteins 1A/1B light chain 3; mRFP, monomeric red fluorescent protein.
suggested that 3-MA and BA inhibited autophagy induced by AFC at different stages.

Furthermore, the effect of AFC on the expression of apoptotic genes in the presence of 3-MA or BA was determined. The expression of cleaved-PARP and cleaved-caspase-3 were increased significantly with combined treatment of AFC and 3-MA or BA compared with either AFC, 3-MA or BA alone in HCT-116 cells (P<0.05; Fig. 4C and D). Similar effects were
evident in the Annexin V-FITC/PI double staining assay, in which combined treatment of AFC and 3-MA or BA increased apoptosis compared with AFC, 3-MA or BA alone in HCT-116 cells (Fig. 4E and F). These data suggest that autophagy...
induced by AFC exerted a suppressive effect on the apoptotic pathways of HCT-116 cells.

**AFC inhibits the activation of the PI3K/Akt/mTOR signaling pathway in HCT-116 cells.** It has been well documented that the PI3K/Akt/mTOR signaling pathway serves a key role in regulating both apoptosis and autophagy through divergent pathways (39). Therefore, the effect of AFC on the PI3K/Akt/mTOR signaling pathway in HCT-116 cells was investigated by western blotting. Treatment with AFC inhibited PI3K phosphorylation, decreased the levels of phospho-Akt, and downregulated phospho-mTOR in a dose-dependent manner (Fig. 5A). The ratios of p-Akt/Akt and p-mTOR/mTOR were significantly decreased following AFC treatment (Fig. 5B). LY294002 is a well-characterized inhibitor of PI3K (40), and the effect of combined AFC (100 µg/ml) and LY294002 (10 µM) on the expression of p-Akt/Akt and p-mTOR/mTOR on HCT-116 cells was investigated by western blotting. The results revealed that the combination of AFC and LY294002 treatment was more effective in decreasing of the ratios of p-Akt/Akt (P<0.001) and p-mTOR/mTOR (P<0.05) compared with AFC or LY294002 alone (Fig. 5C and D). Insulin-like growth factor-I (IGF-I), a PI3K activator, is capable of upregulating PI3K expression, as well as its downstream targets, Akt and mTOR (41). IGF-I treatment (50 ng/ml) significantly increased the phosphorylation of Akt and mTOR (Fig. 5E and F). When cells were pretreated with IGF-I (50 ng/ml), the effect of AFC was significantly attenuated. Furthermore, significant differences in the expression of the LC3-II/LC3-I, cleaved-PARP/procaspase-3 and cleaved-caspase-3/procaspase-3 ratios were observed between AFC treatment alone and the combination of AFC and IGF-I (Fig. 5G and H). These data indicate that the effect of AFC on induction of apoptosis and autophagy is associated with inhibition of the PI3K/Akt/mTOR signaling pathway.

**Discussion**

In the present study, the antitumor effect of AFC was investigated in human colorectal cancer cells. It was demonstrated that AFC inhibited cell proliferation and apoptosis and induced autophagy in a concentration- and time-dependent manner (Figs. 2-4). The effect of AFC on induction of apoptosis and autophagy was demonstrated to occur via inhibition of the PI3K/Akt/mTOR signaling pathway (Fig. 5). Furthermore, autophagy induced by AFC was demonstrated to suppress apoptotic pathways, and inhibition of autophagy by autophagic inhibitors, 3-MA and BA, enhanced the effects of AFC on cytotoxicity and apoptosis of HCT-116 cells (Fig. 4).

In our previous study, two active components of AFC, OA and Eugenol, were identified (12). OA has been demonstrated to exhibit anticancer efficacy against various types of human cancer cells (12,42,43). Our previous study indicated that treatment of human pancreatic Pan-28 cancer cells with OA induced apoptosis via a mitochondrially-mediated apoptotic pathway (13). It was also demonstrated that OA is able to induce protective autophagy in multiple types of cancer cells (30). In the present study, it was indicated that AFC-induced autophagy decreased its effect on induction of apoptosis in human HCT-116 cancer cells. Eugenol has also been demonstrated to possess moderate antitumor activity (44,45), and to trigger apoptosis of breast cancer cells through E2F1/survivin down-regulation (46). The combination of myricetin and methyl Eugenol enhanced the anticancer activity of cisplatin against HeLa cervical cancer cells (47). However, whether a synergistic effect of anticancer activity exists of OA and Eugenol remains unclear and requires further investigation. AFC may contain other anticancer components. Studies are on-going in our laboratory to address the complicated mechanism of the anticancer activity of clove.

Clove is traditionally believed in Chinese medicine to aid gastrointestinal function and to alleviate pain, and historically used to treat nausea, gastric spasm and sore throat (48). In a previous study it was demonstrated that oral administration of aqueous clove (100 µl/mouse/day for 21 weeks) decreased the incidence of tumor development by >50% in a mouse model of benz[a]pyrene (BP)-induced lung carcinogenesis, and that the chemo-preventive effect of clove may be due to inhibition of anti-apoptotic gene expression, including that of Bcl-2, VEGFA and CD44 (10). Our previous study also revealed that clove extracts were capable of inducing apoptosis via mitochondrial pathways in a number of cancer cell lines (12). Considering its low toxicity and its effectiveness, AFC has potential for development as a novel anticancer agent.

In the present study, AFC treatment was demonstrated to enhance autophagy in HCT-116 cells in a dose- and time-dependent manner. Autophagy has been indicated to be induced by anticancer drugs during the induction of apoptosis. Therefore, inducing autophagy-associated cell death of cancer cells may be useful in cancer treatment (49-52). An extract from the tuber of *Amorphophallus* was reported to suppress the growth of proliferation of SGC-7901 and AGS cancer cells by induction of apoptosis and autophagy (53). Fenugreek extract also displayed anticancer effects through induction of autophagy and autophagy-associated death in human T lymphoma jurkat cells (54). The present study suggests that AFC extracts may be used in combination with classical chemotherapeutic agents to achieve an optimized outcome in the treatment of cancer.

In conclusion, the data from the present study indicate that AFC was able to induce typical apoptosis and autophagy in human colorectal cancer HCT-116 cells. Furthermore, the autophagy inhibitors, 3-MA and BA, potentiated the pro-apoptotic activity of AFC in HCT-116 cells. AFC also inhibited the phosphorylation of members of the PI3K/Akt/mTOR signaling pathway. These data may provide scientific rationale for improving the existing understanding of the anticancer mechanism of clove and to further develop AFC as a promising novel anticancer agent used alone or in combination with other chemotherapeutic agents for the treatment of colorectal cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

ML, SC and XL designed the experiments and analysed the data. GZ, DZ, WA and HL performed the experiments. ML, GZ, SC and XL wrote the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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