The Mechanism of Aloe-emodin in the Treatment of Colon Cancer based on Network Pharmacological Analysis

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Abstract

Background: Colon cancer is a malignant gastrointestinal tumor with a high incidence, high mortality and high metastasis in the world. Aloe-emodin is a monomer compound derived from hydroxyanthraquinone. It makes a wide range of anti-tumor effects and exists in Rhubarb, Aloe, and other plants. However, the mechanism of aloe-emodin against colon cancer still not clear. Here, we predict the potential targets and mechanisms of aloe-emodin based on network pharmacology analysis.

Methods: First, determine the intersection target of aloe-emodin and colon cancer, analyze and construct PPI, Gene Ontology, and KEGG pathway analysis. In addition, we selected apoptosis pathways for experimental verification including cell viability determination, cell proliferation, caspase-3 activity determination, DAPI staining, cell cycle determination and western blot to evaluate the apoptosis effect of aloe-emodin on colon cancer cells.

Results: The MTT assay and cell colony experiment showed that AE inhibited cell proliferation ($P < 0.01$). DAPI staining confirmed that AE induced apoptosis. AE activates caspase-3, caspase-9 and Bax and down-regulates the expression of Bcl-2. Furthermore, the expression level of cytochrome C protein increased in a time-dependent manner in the cytoplasm but fell in a time-dependent manner in the mitochondria.

Conclusion: These results indicate that aloe-emodin may induce apoptosis of human colon cancer cells through mitochondrial related pathways.

Background

Colon cancer is among the most common cancers in the world. The global incidence rate is second only to lung cancer and breast cancer, ranking third in the world and the incidence and mortality rate is increasing throughout the year[1]. However, most colon cancers are caused by dietary habits, obesity, lack of physical exercise, smoking and other unfavorable risk factors[2]. Since colon cancer does not appear symptoms until its advanced stage, it brings certain difficulties in diagnosis and treatment[3]. Surgery and chemotherapy are currently the main methods of clinical treatment of colon cancer but chemotherapy drugs often bring side effects to patients[4]. Therefore, there is an urgent need for drugs with small side effects and good curative effects to treat colon cancer to alleviate the suffering of patients. Nowadays, some small molecule compounds have been proven effective for cancer treatment[5–7].

Aloe-emodin is mainly distributed in natural plants such as *Rheum palmatum* L, *Cassia occidentalis* which is a small molecule hydroxy-anthraquinone and more and more evidence shows that Aloe-emodin has a variety of pharmacological activities especially anti-tumor activity [8, 9]. Studies have shown that Aloe-emodin can induce apoptosis of cervical cancer cells which is related to HPV E6, E7 and glucose metabolism[10], Aloe-emodin enhanced the antiproliferative activity of tamoxifen by blocking Ras/ERK and PI3K/mTOR pathways in breast cancer cells[11]. At present, studies demonstrated that Aloe-emodin

have anti-migration and anti-angiogenesis activities in colon cancer cells[12]. In addition, the similar natural active anthraquinone derivative emodin induces cell death by promoting cell cycle arrest and apoptosis of human colon cancer LS1034 cells in vitro and in vivo[13]. However, the mechanism of Aloe-emodin on human colon cancer cells is unclear.

Network pharmacology is a new discipline based on the theory of systems biology[14]. It selects specific signal nodes for multi-target drug molecular design. It mainly emphasizes the multi-channel regulation of signal pathways, improves the therapeutic effect of drugs, reduces toxic side effects and improves the clinical practice of new drugs. Thereby increasing the success rate of clinical trials of new drugs and saving the cost of the drug. Based on network pharmacology analysis, it provides new ideas for the potential mechanism of aloe-emodin in the treatment of colon cancer. In this study, we constructed a protein-protein interaction network by predicted the targets of drugs and diseases which obtained key targets and performed pathway enrichment analysis. In addition, we also used human colon cancer cell HCT116 for experimental verification.

Materials And Methods

1. Chemical structure optimization and ADME evaluation

The structural information of Aloe-emodin was obtained from the PubChem(<https://pubchem.ncbi.nlm.nih.gov/>,Compound CID: 10207) then we evaluate aloe-emodin from the Traditional Chinese Medicine Systems Pharmacology (TCMSP) database (<https://tcmspw.com/tcmsp.php>) through the process of absorption, distribution, metabolism, and excretion of ADME and we were used two indexes, oral bioavailability(OB)and drug-likeness(DL), OB represents the rate and extent of drug absorption into the blood circulation, DL describe the molecular properties affecting pharmaco-dynamics and pharmacokinetics.

2. Aloe-emodin related potential target prediction

The structure of a .sdf file of Aloe-emodin (Compound CID: 10207) obtained from the PubChem database was optimized by Chem3D19.0 for the MM2 force field and saved as a mol2 format file and uploaded to the PharmMapper (<http://www.lilab-ecust.cn/pharmmapper/>) for target prediction, where the species was set to human. Select the result of z'-score > 0 as the target of Aloe-emodin and use the Uniprot database(<https://www.uniprot.org/>) to convert the protein name to the gene symbol and remove unverified target genes.

3.Colon cancer-associated targets prediction

We searched from the following two databases for information about the target of colon cancer. We used colon cancer as a keyword and limited it to searching only Homo sapiens proteins. The first database is

GeneCards (<https://www.genecards.org/>), a comprehensive and comprehensive collection of known or predicted human genes and the other is OMIM (<https://omim.org/>) which is called the online human Mendelian genetic database in Chinese and pass the scores provided by the database and screen for potential targets.

4. Protein-protein interaction (PPI) networks construction

First, we obtained the targets of Aloe-emodin in the two databases and only retained the effective targets related to colon cancer for Wayne analysis. We used Cytoscape 3.8.0 software to construct and analyze the networks. Then, we used the genemania (<http://genemania.org/>) visual network analysis software for the genome in Cytoscape to build a protein-protein interaction network and predict aloe-emodin and colon cancer targets. In the PPI protein network, a node represents a protein and the connection between nodes represents the interaction between protein and protein. Lastly, we selected the top 20 targets of high-node degree as the key targets.

5. GO and KEGG pathway enrichment analysis

We used metascape to enrich the genes shared by Aloe-emodin and diseases, a powerful gene function annotation analysis tool that can quickly help users apply to batch gene enrichment analysis websites through bioinformatics methods (<https://metascape.org/gp/index.html#/main/step1>). At the same time, we restricted the species selection to "Homo sapiens". In this result, $p < 0.05$ is considered to be significant. Then, Heatmap was plotted by <http://www.bioinformatics.com.cn>, an online platform for data analysis and visualization.

Experimental Validation

1. Cell culture

HCT116 cells (Hunan Fenghui Biotechnology Co., Ltd, Hunan, China) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA) in a 37 °C, 5% CO₂ incubator environment.

2. Cell viability assay

MTT assay was used for cell viability assessment. HCT116 cells seeded in 96-well plates at 5000 cells/well. After incubation 24 h treatment with various concentrations of Aloe-emodin (AE was provided by Lot.Y02M11Y16995, Shanghai Yuanye Biological Technology Co., Ltd. Shanghai, China. HPLC $\geq$ 97%) at 24 h, 48 h, or 72 h and 0.5 mg/ml MTT (Sigma, USA) at 37°C under 5% CO₂ for 4 h and then add DMSO 100 μ l (Guangdong Guanghua Technology Co., Ltd., China) to each well. Immediately measure its

absorbance at 490 nm and calculate its cell viability, the percentage of cell viability according to the following formula, OD value of the treated cells/ OD value of the control cell $\times$ 100%. By definition, the viability of the control cells from the untreated cultures was defined as 100%.

3.Colony formation

Measure the effect of cell proliferation through colony formation experiments. The HCT116 was evenly spread in a 60 mm culture dish with 1×10^5 and cultured in complete medium for 24 h, treated with various concentrations of aloe-emodin for 24 h, 48 h, and cultured for 7 days. The cells were stained with crystal violet (Shanghai Aladdin Biochemical Technology Co., Ltd., Shanghai, China) for 15 min and photographed.

4.DAPI staining

DAPI staining can be used to observe apoptosis, penetrate the cell membrane and double-stranded DNA in the nucleus to play a role in labeling. First, HCT116 cells were seeded on 6-well plates at 1×10^5 per well. After 24 hours of culture, the cell was treated with various concentrations of aloe-emodin for 24 h, 48 h. The morphological changes of the cells were observed under a microscope, then fixed with ethanol(Hangzhou Chemical Reagent Company, Hangzhou, China) and PBS (Hangzhou Northrend Biotechnology Co., Ltd., Hangzhou, China,) washed, cells were stained with DAPI (10 mg/mL) (4',6-diamidino-2-phenylindole, Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) for 20 min in the dark, and then photographed by fluorescence microscope(EVOS FL,USA).

5.Caspase-3 Activity Assay

Caspase-3 is a key enzyme in the process of cell apoptosis. Use the caspase-3 activity kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China). for the cultured HCT116 and determine the caspase-3 activity in the Cell lysate according to the protocol provided by the manufacturer. The principle is to change the sequence-specific polymorphism of caspase-3 (Ac-DEVD-pNA) is coupled to the yellow group pNA. When the substrate is cleaved by caspase-3, the yellow group pNA is released, and then the caspase-3 activity is measured by the absorbance value of 405 nm in the microplate reader (Synergy H1, Biotek, USA).

6.Cell cycle by flow cytometry

The distribution of cells in the cell cycle is assessed by flow cytometry. HCT116 was spread on a 6-well plate and cultured overnight. It was treated HCT116 cells with different concentrations of Aloe-Emodin for 24 h and 48 h. The adherent cells were digested with trypsin containing EDTA (Gibco, USA), collected in complete DMEM medium (Gibco, USA), centrifuged, washed with PBS (Hangzhou Northrend

Biotechnology Co., Ltd., Hangzhou, China), fixed with ethanol at -20 °C overnight, treated with PI/Rnase (BD Biosciences, San Diego, USA), and analyzed by Beckman flow cytometer (Beckman, USA) cell cycle.

7. Western blotting

HCT116 cells were seeded in a 6-well plate, treated with different concentrations of aloe-emodin for 24 hours, washed with pre-chilled PBS, and then lysed in lysis Solution (Shanghai Beyotime Biotechnology Co., Ltd., Shanghai, China) on ice for 30 minutes then collected the cells. The protein concentration was established by the BCA method. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the PVDF membrane. The membrane was blocked with 5% skimmed milk powder (Shanghai Yuanye Biological Technology Co., Ltd. Shanghai, China.) and then with the primary antibody overnight at 4 °C. Then the membrane was washed in TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at 37°C. Finally, the immunoreactive band by using ECL (Affinity Biosciences, Changzhou, China) exposed on the Chemiluminescence digital imaging system (Bio-Rad, USA). Then use Image J 1.51 k software to qualitatively and quantitatively analyze the protein bands.

8. Statistical analysis

All data were presented as mean ± SD, and statistical analysis was carried out by GraphPad Prism 8 software. The one-way ANOVA analysis was implemented to demonstrate differences between groups (P < 0.05).

Results

1. Chemical structure optimization and ADME evaluation

The structural information of aloe-emodin was obtained from PubChem(Fig. 1), and the relevant information of ADME was obtained from TCMSP. The threshold values of the two indexes show in Table 1. This shows that aloe-emodin has good bioavailability and oral utilization degree.

Table 1
OB and DL properties of AE

Name	MW	OB(%)	DL
aloe-emodin	270.25	83.38	0.24

2. Target prediction

Based on the potential of Aloe-emodin to exert pharmacological effects through multiple targets, we collected the targets through PharmMapper[15] and the Uniprot database, which deleted duplicate unverified targets, and obtained 404 targets of aloe-emodin. In addition, we have screened the disease targets and obtained 845 disease gene targets according to the scores provided by the OMIM Genecards database. In order to improve the specificity of the target, we collected the targets of diseases and drugs and obtained 43 targets that selected the intersection genes as the data for further analysis.

We construct a PPI network based on 43 targets and the protein interaction network can simply indicate the interaction between protein and protein[16]. The result is shown in Fig. 2. The larger the node, the greater the interaction between proteins and the stronger the degree, the straight line indicates the connection between protein and protein. Then we use Cytoscape to construct a target network according to the degree of protein interaction and select protein gene targets with a higher degree of action (Fig. 3C) for KEGG pathway analysis (Fig. 3A) and GO enrichment analysis (Fig. 3B). Among the related pathways of colon cancer, the potential pathways that are significantly related to the aloe-emodin target have been identified, such as Pathways in cancer, Colorectal cancer, Proteoglycans in cancer, PI3K-Akt signaling pathway, Prolactin signaling pathway, Neurotrophin signaling pathway, FoxO signaling pathway, ErbB signaling pathway, etc. The results showed that the Pathways in cancer of the aloe-emodin target has the lowest p-value and the highest overlap. In the drug treatment of tumors, inducing tumor cell apoptosis plays an important role. Therefore, we selected the apoptotic pathway in the Pathways in cancer pathway for experimental verification (Fig. 4).

3.Effect of aloe-emodin on the viability of HCT116 colon cancer cells

First, the effect of aloe-emodin on cell viability was measured by MTT. The results showed that the cell viability of aloe-emodin on HCT116 decreased with increasing dose and time and the decreasing trend of vigor at 48 h and 72 h was consistent (Fig. 5A). In addition, we further demonstrated the anti-proliferative effect of aloe-emodin on HCT116 cells through colony formation experiments, and the results were also interesting. Colony formation experiments further show that when the drug concentration increases and the time is prolonged then the cell mass decreases significantly which indicating that aloe-emodin inhibits cell proliferation and is dose-dependent (Fig. 5B). Based on the data results, we used an inverted fluorescence microscope to observe the cell morphology of HCT116 cells treated with aloe-emodin for 48 h which most of the cells fell off the culture plate and compared with untreated normal cells that the cells the apoptotic morphology increase such as membrane blistering and the morphology of the cells exhibits shrinkage under the stimulation of drug administration(Fig. 5C).

4.Effect of aloe-emodin on apoptosis of HCT116 colon cancer cells

According to the above results, we further observed the morphological changes of cell apoptosis by DAPI staining (Fig. 6A). There are consistent with the results of colon cancer cells treated with drugs that vacuoles appear in the cytoplasm and apoptotic bodies appear. We carried out a caspase-3 activity assay to further determine the aloe-emodin induced apoptosis of HCT116 colon cancer cells. The results showed that the caspase-3 activity in HCT116 treated with aloe-emodin for 24 h showed an upward trend and the treated for 48 h was significantly higher than 24 h (Fig. 6C). Subsequently, we conducted cell cycle measurement and found that the cell arrest induced by aloe-emodin was in the G0/G1 phase (Fig. 6B). To further clarify the mechanism of aloe-emodin induced apoptosis, we also performed western blot analysis. Compared with the control group, the protein expression levels of Bcl-2, caspase-3, caspase-9 and cytochrome C in Mitochondria in HCT116 cells treated with aloe-emodin decreased, while Bax, cleaved caspase-3, cleaved caspase-9 and cytochromes C in the cytoplasm was higher than that of the control group. This implies that the anti-colon cancer mechanism of aloe-emodin may be related to the activation of mitochondrial pathways.

Discussion

In the past few decades, many studies have reported that natural medicine has many advantages in the treatment of cancer [17–19]. Aloe-emodin is an anthraquinone compound widely found in plants such as *Rheum palmatum* L, Aloe, and has significant anti-inflammatory, analgesic, and anti-swelling effects[20]. Aloe-emodin has anti-cell proliferation and inducing apoptosis effects in many cancer cells[21, 22]and aloe-emodin can cross HT-29 cellular membranes and pass through the intestinal layer[23]. So, we boldly guess whether aloe-emodin has the effect of treating colon cancer? However, few studies have determined the key targets and pathways of aloe-emodin for human colon cancer.

Nowadays, more and more people use biological information technology to study the interaction between human diseases and drug targets[24] and pathways and predict the biological activity of drugs and unknown pharmacological effects based on the screening data which improves research efficiency and saves time and cost [25]. In this study, we used bioinformatics for the first time to analyze and predict the potential targets of aloe-emodin and colon cancer, and understand the potential mechanism of drug interaction with the body from a holistic perspective. First, determine the intersection target of aloe-emodin and colon cancer, analyze and construct PPI, Gene Ontology, and KEGG pathway analysis. Subsequently, the role of aloe-emodin and colon cancer related targets and pathways was further explored by constructing a target-pathway interaction network diagram. In addition, we selected apoptosis pathways for experimental verification considering the targets and pathways and adopted several in vitro experimental methods including cell viability determination, cell proliferation, Caspase-3 activity determination, DAPI staining, cell cycle determination, and western blotting to evaluate the apoptosis effect of aloe-emodin on colon cancer cells and to reveal the treatment of colon cancer by aloe-emodin. It provides new ideas for revealing the mechanism of aloe-emodin in the treatment of colon cancer.

The results showed that we obtained 43 potential targets based on predicted the targeting effect of aloe-emodin on colon cancer through network pharmacology. GO and KEGG pathway analysis showed that aloe-emodin has a wide range of anti-tumor activities and can regulate a variety of tumor-related pathways. Many factors in cancer development regulate tumor development which may promote or inhibit tumors. They provide two obvious targets for therapeutic intervention in all cancers which non-regulated proliferation and inhibition of apoptosis are the core of all tumor development[26]. Targeted inhibition of tumor growth and induction of tumor cell apoptosis together provide a potential platform for studying tumor progression[27]. Aloe-emodin inhibits the growth of HeLa cells in a dose-dependent manner within a certain concentration range[28], acts on PKC isozymes to induce lung cancer cell apoptosis[29], inhibit the protein levels and activities of matrix metalloproteinase-2 induce human tongue cancer SCC-4 cell apoptosis [30]and also induces apoptosis of T24 human bladder cancer cells through the p53-dependent apoptosis pathway[31]. These multiple mechanisms of action may be responsible for the anticancer effects of aloe-emodin on different types of cancer. We construct a target-pathway interaction network diagram to speculate the potential mechanism of aloe-emodin in the treatment of colon cancer. Cell apoptosis refers to the autonomous and orderly death of cells to maintain a stable internal environment which is divided into exogenous pathways and endogenous pathways[32]. Therefore, we performed KEGG analysis based on the predicted targets, and selected apoptotic pathways related to colon cancer pathways for verification. The results showed that cell apoptosis through the mitochondrial pathway after treatment with aloe-emodin. The mitochondrial pathway is an endogenous pathway of the cell which is regulated by the interaction between the Bcl-2 family and mitochondria[33]. Mitochondria are the control center of cell life activity and the control center of apoptosis. We found that aloe-emodin can effectively inhibit the proliferation of colon cancer cells which present certain concentration-dependent and morphological changes accompanied by apoptosis such as membrane blistering, cell needle tip contraction. The cells are blocked in the G0 / G1 phase and apoptotic cells increased significantly and DAPI staining further confirmed the above-mentioned apoptotic effect. In addition, Caspase-3 plays an irreplaceable role in cell apoptosis and is the most important terminal splicing enzyme in cell apoptosis. This study proved that caspase-3 is activated and participates in cell apoptosis induced by aloe-emodin. Subsequently, we observed the release of caspase dependent Bcl-2 family and cytochrome C in the process of aloe-emodin induced apoptosis by western blotting. The Bcl-2 family is divided into pro-apoptotic factors and anti-apoptotic factors[34]. When the mitochondrial pathway is activated, anti-apoptotic proteins are oligomerized and inserted into mitochondria[35], causes the permeability of the mitochondrial membrane to change, the transmembrane potential is lost and cytochrome C and other proteins are released into the cytoplasm. The results showed that the expression of the pro-apoptotic factor Bax protein in the Bcl-2 family increased while the expression of the anti-apoptotic factor Bcl-2 protein decreased after treatment with aloe-emodin. The pro-apoptotic Bax protein is the main effector of mitochondrial permeabilization during apoptosis[36]. In addition, our results showed that the expression level of cytochrome C protein increased in a time-dependent manner in the cytoplasm but decreased in a time-dependent manner in the mitochondria. The release of cytochrome C is a key step in the mitochondrial apoptosis pathway[37]. At the same time, the activity of caspase-3 increases, also caspase-9 which may be related to the cytochrome C released into the cytoplasm. The

release of cytochrome C from the mitochondria to the cytoplasm is the beginning of the mitochondrial apoptosis pathway of cells. Caspase-3 and caspase-9 are executors and participants of the mitochondrial pathway. Cytochrome C is released into the cytoplasm initiator caspase-9 which then cleaves and activates Caspase-3 the effector caspases to execute cell killing [38]. Therefore, our results provide preliminary evidence that aloe-emodin may induce apoptosis in human colon cancer cells through mitochondrial-related pathways which further supports the anti-colon cancer effect of aloe-emodin. However, there are some limitations. For example, due to cost and time constraints, this study only verifies the key molecules in the mitochondrial apoptosis pathway. Secondly, some targets with low enrichment scores were not studied in this study. Besides, it is only verified in vitro experiments, not in vivo experiments.

Conclusion

In this study, we used network pharmacology analysis to predict the potential targets and mechanisms of aloe-emodin, constructed a PPI network, and exploring the role of aloe-emodin in the treatment of colon cancer through KEGG and GO enrichment analysis, and confirmed that aloe-emodin induces apoptosis of human colon cancer cells in vitro experiments. MTT determined the effect of aloe-emodin on the survival rate of colon cancer cells. The cell colony experiment further showed the effect of aloe-emodin on colon cancer cells. DAPI staining and caspase-3 activity further evaluated the Aloe-emodin-induced apoptosis of colon cancer cells. Besides, western blotting detected that aloe-emodin activates caspase-3, caspase-9, and Bax and down-regulates the expression of Bcl-2. The expression level of Cytochrome C protein increases in the cytoplasm in a time-dependent manner while in the mitochondria. These results indicate that aloe-emodin can induce apoptosis of human colon cancer cells through mitochondrial-related pathways.

Abbreviations

AE: Aloe-emodin; OB: oral bioavailability; DL: drug-likeness; PPI: Protein-protein interaction; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; PBS: Phosphate buffer saline; ANOVA: Analysis of variance.

Declarations

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Authors' contributions

JDX, DSF and YLY conceived, designed the research, wrote the manuscript and conducted research and data analysis. MZJ and RYP reviewed the manuscript and revised and improved the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in the manuscript.

Disclosures

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Competing interests

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

All listed authors have actively participated in the study and have read and approved the submitted manuscript.

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Figures

Figure 1

The structural information of Aloe-emodin

Figure 2

Network construction. (A)Veen diagram of candidate targets of diseases and drugs and obtained 43 collective targets. (B) The interactive PPI network of Aloe-emodin and disease targets. The relationship between the size of circles and degree value of targets is linear positive correlation. (C). Select the top 20 targets from degree value of targets. 3. Network pharmacology analysis

Figure 3

(A)The enrichment analysis of the KEGG signaling pathways. (B)The GO enrichment analysis of potential targets. (C)Construct a target network according to the degree of protein interaction and select protein gene targets with a higher degree of action.

Figure 4

Predict the mechanism of aloe-emodin induced apoptosis of HCT116

Figure 5

Effect of aloe-emodin on the viability of HCT116 colon cancer cells. (A) Cell viability was determined by MTT assay after treated with aloe-emodin for 24, 48 and 72h. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Control. (B) Colony formation experiments of HCT116 colon cancer cells treated with aloe-emodin for 24h, 48h. ** $P < 0.01$ versus Control. (C) Fluorescence microscope to observe the cell morphology of HCT116 cells treated with aloe-emodin for 48h ($\times 20$ magnification).

Figure 6

Effect of aloe-emodin on apoptosis of HCT116 colon cancer cells. (A) DAPI staining ($\times 20$ magnification), 40μM 80μM for 24h ($\times 40$ magnification) and the white arrow points to the salient features of apoptotic cells. (B) The cell cycle is assessed by flow cytometry. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus Control. ### $P < 0.001$ versus 20μM. (C) Caspase-3 activity assay. Data are expressed as mean $\pm$ SD. * $P < 0.05$ versus Control.

Figure 7

Treatment with aloe-emodin for 24h and western blotting analysis the protein expression levels of Bax, Bcl-2, caspase-3, caspase-9, cleaved caspase-3 and cleaved caspase-9. Data are expressed as mean $\pm$ SD. * P <0.05, ** P <0.01, *** P <0.001 versus Control.

Figure 8

Treatment with aloe-emodin for 24h, mitochondria and cytoplasm were separated by mitochondrial/cytoplasmic separation kit. Western blotting assessments the expression of Cytochromes C in mitochondria and cytoplasm. Data are expressed as mean $\pm$ SD. *P<0.05 versus Control.