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Ethanolic extract of *Cordyceps cicadae* exerts antitumor effect on human gastric cancer SGC-7901 cells by inducing apoptosis, cell cycle arrest and endoplasmic reticulum stress

Hongqing Xie, Xiaotong Li, Yajie Chen, Mingzi Lang, Zhangfei Shen, Liangen Shi*

College of Animal Sciences, Zhejiang University, Hangzhou 310058, China

蟬草:麥角菌科蟲草屬真菌，會寄生在蟬蛹或者是幼蟲身上，待將蟲體內營養吸收盡後使其死亡，然後將其屍體變成菌核，並在屍體前段長出子座

ARTICLE INFO

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Cordyceps cicadae
Antitumor activity
Caspase-dependent apoptosis
Cell cycle arrest
Endoplasmic reticulum stress
SGC-7901 cells

ABSTRACT

民族藥理學相關性

Ethnopharmacological relevance: *Cordyceps cicadae* (Miq.) Massee is a traditional Chinese medicine that has been used for approximately 1600 years in China. *C. cicadae*, a member of the *Cordyceps* genus, exerts a therapeutic effect on many diseases, such as cancer.

Objective: This study aimed to evaluate the antineoplasmic activity of *C. cicadae* and to identify its molecular mechanism of cell death.

Materials and methods: The toxicity of the ethanolic extract of *C. cicadae* (EEC) against different cancer cell lines was determined through MTT assay. Human gastric cancer SGC-7901 cells were treated with EEC for 48 h. Cell morphology was examined by using an Olympus phase-contrast microscope. The cell apoptosis was quantified through Annexin V-FITC/PI staining. Cells were stained with PI and then subjected to flow cytometry for the investigation of cell cycle status. Cells were subjected to mitochondrial membrane potential (MMP) assay after incubation with JC-1 probes and to intracellular Ca^{2+} measurement through flow cytometry after incubation with Fluo-3 AM fluorescent probes. Western blot analysis was conducted to quantify the expression of proteins related to apoptosis, cell cycle and endoplasmic reticulum stress. High-performance liquid chromatography (HPLC) analysis was performed to analyse the biological activity components of EEC.

Results: EEC suppressed the proliferation of SGC-7901 cells and induced the development of abnormal morphological features in a dose-dependent manner. Flow cytometry results indicated that EEC treatment caused cell apoptosis and arrested the cell cycle in the S phase. In addition, EEC treatment triggered MMP depolarization and Ca^{2+} overloading in the cytosol of SGC-7901 cells. Western blot analysis demonstrated that EEC increased Bax, AIF, caspase-8, caspase-6 and caspase-3 activities and decreased Bcl-2 activity. The release of cytochrome c from mitochondria was associated with mitochondrial dysfunction, which was caused by the activation of the cell surface receptor Fas and the cleavage of PARP. EEC-induced S phase arrest was associated with the up-regulation of E2F1, cyclin A2, cyclin E and p53 expression levels and the down-regulation of CDK2 expression. In addition, EEC increased the expression of endoplasmic reticulum stress-related proteins, such as calpain-1, caspase-12 and caspase-9. HPLC assay results suggested that EEC contained adenine, uridine, adenosine and N6-(2-Hydroxyethyl)-adenosine.

Conclusion: EEC inhibited the proliferation of SGC-7901 cells by inducing caspase-dependent apoptosis, arresting the cell cycle in the S phase and increasing endoplasmic reticulum stress. This study revealed that *C. cicadae* is a potential natural source of anticancer drugs.

1. Introduction

Cancer is a fatal disease caused by the abnormal growth regulation and proliferation of cells (Markert, 1968). Gastric cancer is the second most common cancer and the second main cause of cancer-related deaths in China (W.Q. Chen et al., 2018). Surgery, chemotherapy and radiotherapy are the primary treatments for cancer. Surgery can effectively treat early-stage tumours, and chemotherapy is frequently employed after surgical treatment. Nevertheless, drug resistance and side effects have reduced the efficacy of orthodox anticancer drugs. A

* Corresponding author.
E-mail addresses: xiehongqing@zju.edu.cn (H. Xie), lixiaotong@zju.edu.cn (X. Li), 11417010@zju.edu.cn (Y. Chen), 21617032@zju.edu.cn (M. Lang), szf@zju.edu.cn (Z. Shen), slgsilk@zju.edu.cn (L. Shi).

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cancer treatment strategy that combines traditional Chinese medicine and Western medicine may exhibit attenuated side effects and improved curative effects (Wu et al., 2005). Cancer treatment can be achieved through the regulation of immune function; induction of cancer cell apoptosis: elimination of multidrug-resistant cancer cells; regulation of cell signal transduction; and inhibition of vascular tumour growth and cancer cell infiltration, migration and metastasis (Ruan et al., 2006). Numerous *Cordyceps* species exhibit antitumor activities (Kuo et al., 1998). Fermentation broth from *Cordyceps militaris* cultures suppresses the proliferation of murine colorectal adenocarcinoma CT26 cells (Lin and Chiang, 2008). Jiangxienone, which is extracted from *Cordyceps jiangxiensis*, exhibits potent cytotoxic effects against human gastric adenocarcinoma SGC-7901 cells and human lung carcinoma A549 cells (Xiao et al., 2012a). The aqueous extract of *C. militaris* suppresses the growth of human leukaemia U937 cells by inducing apoptosis (Park et al., 2005). Moreover, *C. sinensis* inhibits the multiplication of human breast cancer MCF-7 cells and human hepatoma cancer HepG2 cells (Wu et al., 2007).

Cordyceps cicadae (Miq.) Massee is used as a tonic food and traditional herbal medicine. *Cordyceps cicadae* is composed of dried *Cicada flammata* larvae that have died and stiffened from infection with *Paecilomyces cicadas*. *C. cicadae* was first mentioned in "Lei's Treatise on Preparing Drugs", which was written in the fifth century A.D (Hsu et al., 2015). *C. cicadae* has a high nutritional value and specific therapeutic effects. Its bioactive constituents include nucleosides, cordycepin, polysaccharides, beauvericin, myriocin (ISP-1), ergosterol and peroxides.

In herbal medicine prescriptions and folk medicine, *C. cicadae* is mainly used for immunoregulation and for the treatment of chronic kidney disease, convulsion symptoms, morbid night crying of babies, hot flashes, cough caused by pneumonia and cancer (Jiangsu New Medical College, 1977). The aqueous methanolic (50%) extracts of *C. cicadae* ascocarps portion promote the growth of human mononuclear cells by activating phytohemagglutinin, whereas the methanol extracts of *C. cicadae* insect-body portion suppress the multiplication of human mononuclear cells (Weng et al., 2002). Ergosterol peroxide isolated from *C. cicadae* suppresses proliferation and activation signals in primary human T lymphocytes (Kuo et al., 2003).

The components of *C. cicadae* also demonstrate antitumor activity. For example, beauvericin compounds extracted from *C. cicadae* show remarkable cytotoxicity against HepG2 and HepG2/ADM cells with multiple-drug resistance (J. Wang et al., 2014). Polysaccharides isolated from *C. cicadae* suppress the growth of S180 sarcoma *in vitro* (Ukai et al., 1983). The water extract of *C. cicadae* inhibits the multiplication of human hepatocellular carcinoma MHCC97H cells through the induction of G2/M phase arrest instead of through the induction of apoptosis (H. Wang et al., 2014). Aqueous decocting and alcohol soaking are two traditional methods used for the preparation of Chinese medicine. Information on the antineoplastic activity of the ethanolic extract of *C. cicadae* (EEC) remains limited. Consequently, the mechanisms involved in the anticancer effects of EEC warrant further study. We characterised the inhibitory effects of EEC on the proliferation of different cancer cell lines. We found that EEC exerts remarkable antitumor effects. Moreover, EEC treatment results in the development of SGC-7901 cells with abnormal morphology. Through flow cytometry, we found that EEC promotes apoptosis, increases cytosolic Ca^{2+} levels and arrests the cell cycle in the S phase. These effects are related to mitochondrial transmembrane potential loss and abnormal endoplasmic reticulum stress. Our immunoblot assay results show that the effects of EEC are mediated by key regulators in the signalling pathways of apoptosis, cell cycle and endoplasmic reticulum stress. Our data provide experimental evidence for the potential use of *C. cicadae*-derived components as sources of anticancer drugs.

2. Materials and methods

2.1. Materials and reagents

DMEM (high glucose) and 0.25% Trypsin were purchased from Thermo Scientific HyClone (Logan, UT, USA). FBS was obtained from Gibco Industries Inc. (Grand Island, NY, USA). MTT and DMSO were obtained from Sigma-Aldrich (St. Louis, Mo, USA). Annexin V-FITC/PI Apoptosis Analysis Kit was obtained from Tianjin Sungene Biotech Co., Ltd (Tianjin, China). Cell Cycle Detection Kit was purchased from Keygen Biotech Co., Ltd (Nanjing, China). JC-1 assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

For western blotting analysis, the primary rabbit antibodies against β-actin, AIF, Bcl-2, Bax, phosphorylated retinoblastoma protein (P-Rb) and Calpain-1 were purchased from HUABIO Biotechnology (Hangzhou, China). The primary antibodies against CDK2, Caspase-3, Caspase-8, Caspase-9, Caspase-12, Cytochrome C (mitochondria), Cytochrome C (cytoplasm), E2F1, poly (ADP-ribose) polymerase (PARP), Cleaved-PARP, p53, Cyclin A2, Cyclin E were purchased from Cell Signalling Technology (Beverly, MA, USA). Anti-rabbit lgG horseradish peroxidase-conjugated secondary antibody was purchased from Abcam (Cambridge, MA, USA).

Adenine, Uridine, Adenosine, N6-(2-Hydroxyethyl)-Adenosine (all HPLC-grade) were obtained from Aladdin (Shanghai, China).

2.2. Preparation of EEC

C. cicadae was obtained from Huqingyutang Drugstore in Hangzhou and was identified by Professor Zhiyi Ye. A voucher specimen (No. 20170826) was deposited in the Sericulture Bioreactor Lab, College of Animal Sciences, Zhejiang University, China. *C. cicadae* samples were dried, ground and passed through 80-mesh sieves. 40 g of *C. cicadae* powder was extracted with 95% ethanol (1:10, w/v) and refluxed three times for 2 h at 100 °C. The extract was cooled to 25 °C, subjected to vacuum filtration and evaporated in a rotary evaporator under low pressure, lyophilized with LABCONCO Freeze Dryer (Kansas City, MO, USA), obtained 4.86 g crude ethanolic extract. Next, the crude ethanolic extract was dissolved in distilled water (1:10, w/v). The solution was centrifuged at 5000 × *g* for 30 min. The precipitate was discarded from the solution. Then, the supernatant was filtered with double filter paper and lyophilized with freeze dryer. Thus, 4.19 g of the ethanolic extract of *C. cicadae* (EEC) was obtained.

2.3. Cell lines and culture

Lung cancer H1299, hepatocellular carcinoma HepG2, lung adenocarcinoma A549, cervical cancer HeLa, human gastric carcinoma SGC-7901 and human embryonic kidney HEK293 cells were obtained from the Cell Research Institute of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% (v/v) FBS. The aseptic cultures of the cells were maintained in an incubator (Thermo Electron Corporation, MA, USA) under a humid 5% CO_2 atmosphere at 37 °C.

2.4. Cytotoxicity assay

The MTT assay was conducted to detect the cytotoxic effects of EEC on five different cancer cell lines and HEK293 cells. Cells were seeded in 96-well plates (Costar Corning, Rochester, NY) at the density of 6×10^3 cells/well and cultured for 24 h to allow adherence. Next, 200 μL of DMEM medium-soluble EEC with different concentrations was added to each well. Five wells were incubated with each concentration. After 48 h of treatment, the EEC medium was removed from the wells, and 200 μL of 0.5 mg/mL MTT solution was added. The cells were then cultivated for an additional 4 h at 37 °C. After the removal of MTT medium, 150 μL of DMSO was added to the each well. The cells

were shaken at low speed for 5 min. Absorbance was measured at 490 nm (A_{490}) by using a multimode reader (Thermo Electron Corporation, MA, USA). Wells without cells were used as blanks. Growth inhibition rate was calculated as follows:

$$\text{Cell viability (\%)} = (A_{490\ \text{treated}} - A_{490\ \text{blank}})/(A_{490\ \text{control}} - A_{490\ \text{blank}}) \times 100\%.$$

2.5. Morphological observation

Cell morphology was evaluated by using an Olympus phase-contrast microscope. SGC-7901 cells were seeded in six-well plates (Costar Corning, Rochester, NY) at the density of 1×10^5 cells/well. Each well contained 2 mL of culture medium. The cells were then treated with different EEC concentrations for 48 h. Cell morphology was observed through microscopy under 20× magnification.

2.6. Cell apoptosis assay

SGC-7901 cells were seeded in six-well plates at the density of 1×10^5 cells/well. Each well contained 2 mL of culture medium. The cells were incubated for 24 h for the detection of EEC-induced apoptosis. The cells were then incubated with EEC for 48 h, collected and rinsed with PBS and resuspended in 300 μL of binding buffer. Next, 5 μL of Annexin V-FITC was added to the cell suspensions. The cell suspensions were incubated in the dark for 10 min. Then, 5 μL of PI was added to the cell suspensions. The cell suspensions were then incubated for an additional 15 min in the dark. Cell apoptosis was detected by using a BD FACSVerse flow cytometer.

2.7. Cell cycle assay

Flow cytometry was conducted to determine whether SGC-7901 cells underwent cell cycle arrest after EEC treatment. Briefly, cells were collected after 48 h of exposure to EEC, rinsed three times with PBS, immobilised with 70% ice-cold ethanol overnight and washed thrice with PBS. Next, 100 μL of RNase A was added to the cells. The cells were then incubated for 30 min at 37 °C and were resuspended with 400 μL of PI. The cells were maintained at 4 °C for 30 min away from the light. The distribution of cells in different cell cycle phases was detected by using a BD FACSVerse flow cytometer as previously described (Y.J. Chen et al., 2018).

2.8. Mitochondrial membrane potential assay

As previously mentioned, flow cytometry was conducted by using a JC-1 probe to determine the potential role of EEC in changes in mitochondrial membrane potential (MMP) (X.T. Li et al., 2018). Briefly, after treatment with EEC, SGC-7901 cells were collected, washed thrice, maintained in 0.5 mL of JC-1 working solution for 20 min at 37 °C, cleaned, suspended in DMEM medium without FBS and analysed by using a BD FACSVerse flow cytometer.

2.9. Intracellular Ca^{2+} measurement

Alterations in intracellular Ca^{2+} were analysed through flow cytometry with a Fluo-3 AM probe (Beyotime Biotechnology, Jiangsu, China) (Shen et al., 2018). SGC-7901 cells were rinsed thrice with PBS and maintained in Fluo-3 AM solution (1 μM diluted by normal medium) for 30 min at 37 °C. Then, the cells were washed, incubated in PBS and analysed by using a BD FACSVerse flow cytometer.

2.10. Western blot analysis

Protein extracted from SGC-7901 cells was subjected to immunoblot assay with the indicated antibodies for the analysis of cell death-related proteins (Shen et al., 2017). SGC-7901 cells were collected after 48 h of incubation with EEC and were rinsed thrice with cold PBS. A one-step animal cell active protein extraction kit (Sangon Biotech, Shanghai, China) was used to extract the total protein of SGC-7901 cells after EEC treatment. The extracted protein was packed and stored at −20 °C. Protein contents were measured by using a BCA protein assay kit (Sangon Biotech, Shanghai, China). In Western blot analysis, 20 μg of proteins were separated through 10% SDS-PAGE and electrotransferred to PVDF membranes (Millipore, USA). The membranes were blocked in blocking buffer at 25 °C for 2 h and incubated with the corresponding primary antibodies at 4 °C overnight. Subsequently, the membranes were rinsed thrice with TBST buffer and incubated for 1 h with secondary antibodies under low-speed oscillation at room temperature. The blots were washed with TBST buffer thrice and detected by using an Immobilon Western Chemiluminescent HRP substrate. Images of the blots were acquired, and densitometric analysis was performed by using ChemiScope 3300 Mini (Clinx Science Instruments, Shanghai, China).

2.11. Biological activity component analysis of EEC

Total sugar was quantified through the phenol-sulfuric acid method as previously described (Y.J. Chen et al., 2018). Nucleoside content was quantified through high-performance liquid chromatography (HPLC) analysis. EEC and reference compounds were dissolved in 2% methanol. Methanolic solutions were diluted to the appropriate concentrations and filtered by using a 0.22 μm membrane filter. Separations were carried out by an Agilent series 1200, equipped with a G1322A vacuum degasser, G1311A pump, G1329A autosampler, G1314B detector, G1316A Thermostatted Column Compartment and SepaxHP-C18 column (150 mm × 4.6 mm, 5 μm) operating at 30 °C. The mobile phase comprised 1.0% acetic acid (v/v) (A) and methanol (B) and was applied in gradient mode under the following conditions: 0–15 min, 2–3% B; 15–30 min, 3–4% B. The flow rate was 1.0 mL/min. The effluent was monitored at 254 nm, and the injection volume was 5 μL.

2.12. Statistical analysis

Statistical analysis was performed with SPSS statistical software version 10.0 and GraphPad Prism 6.0. Data were shown as mean ± standard deviation. Student's *t*-test was performed for statistical analysis. In this study, differences were considered significant if $p < 0.05$ and extremely significant if $p < 0.01$.

3. Results

3.1. EEC inhibited the proliferation of SGC-7901 cells

The cytotoxicity of EEC against five different cancer cell lines and human embryonic kidney HEK293 cells was determined through the MTT assay. EEC showed cytotoxicity against SGC-7901, H1299, HeLa, HepG2 and A549 cells at the concentration of 1600 μg/mL (Fig. 1A-E). The viability of SGC-7901 cells was lower than that of four other types of cancer cells and decreased as EEC concentration increased (Fig. 1E). The viability of HEK293 cells increased under treatment with low concentrations of EEC (25 or 50 μg/mL). Treatments with 100–800 μg/mL EEC, however, cell inhibition rates were not significantly affected. Treatment with a high concentration of EEC (1600 μg/mL) reduced the viability of HEK293 cells (Fig. 1F). The IC50 values of EEC against H1299, HepG2, A549, HeLa and SGC-7901 cells were 1990, 1201, 570.4, 550.9 and 121.4 μg/mL, respectively. These results indicate that EEC has optimal antitumor activity against SGC-7901 cells.

The morphological changes exhibited by SGC-7901 cells were evaluated to determine whether EEC can inhibit cell growth. Cells cultured under normal conditions appeared polygonal in shape and grew closely attached to cell plates (Fig. 2A). After EEC treatment,

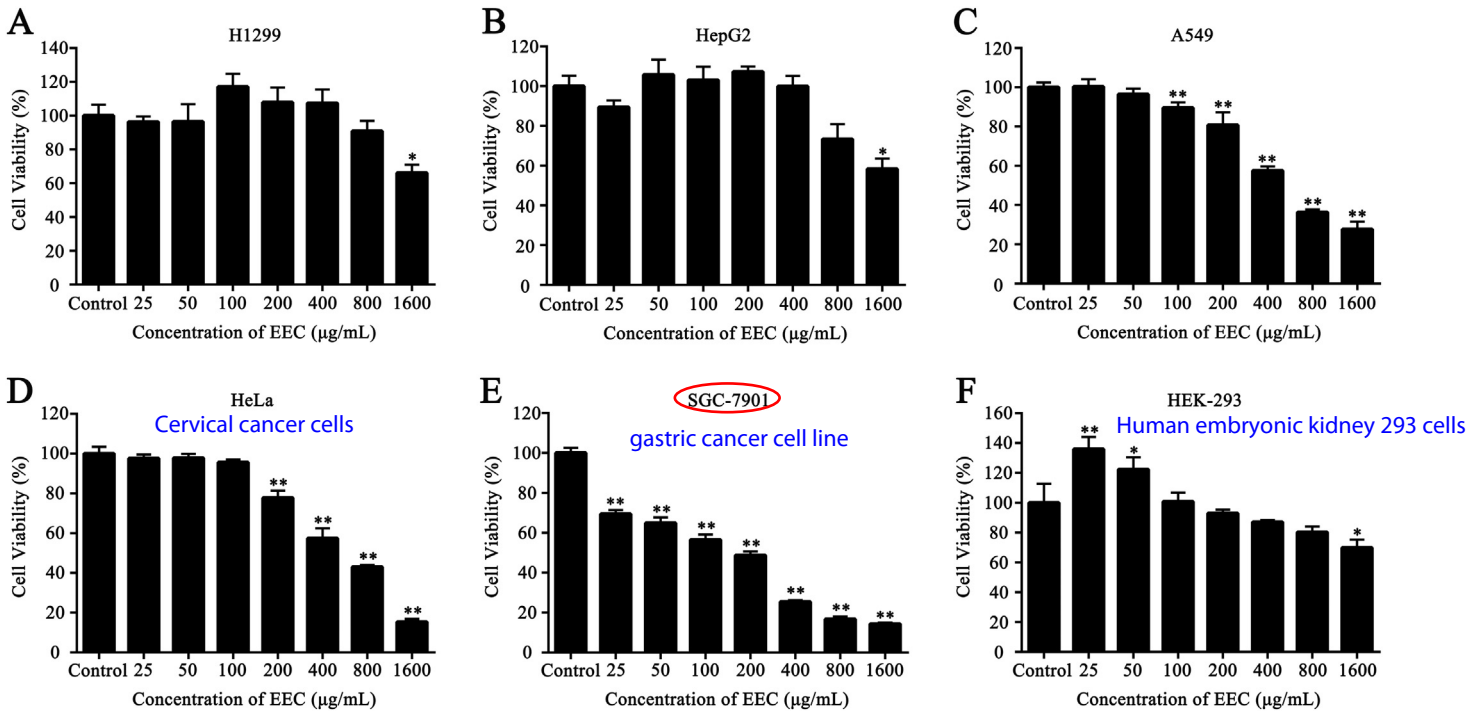


Fig. 1. Estimation of growth inhibition by MTT assay at different doses of EEC treatment.

Symptoms do not mention gastric cancer
kidney; lung --> other kidney or lung cell lines

More gastric cancer cells

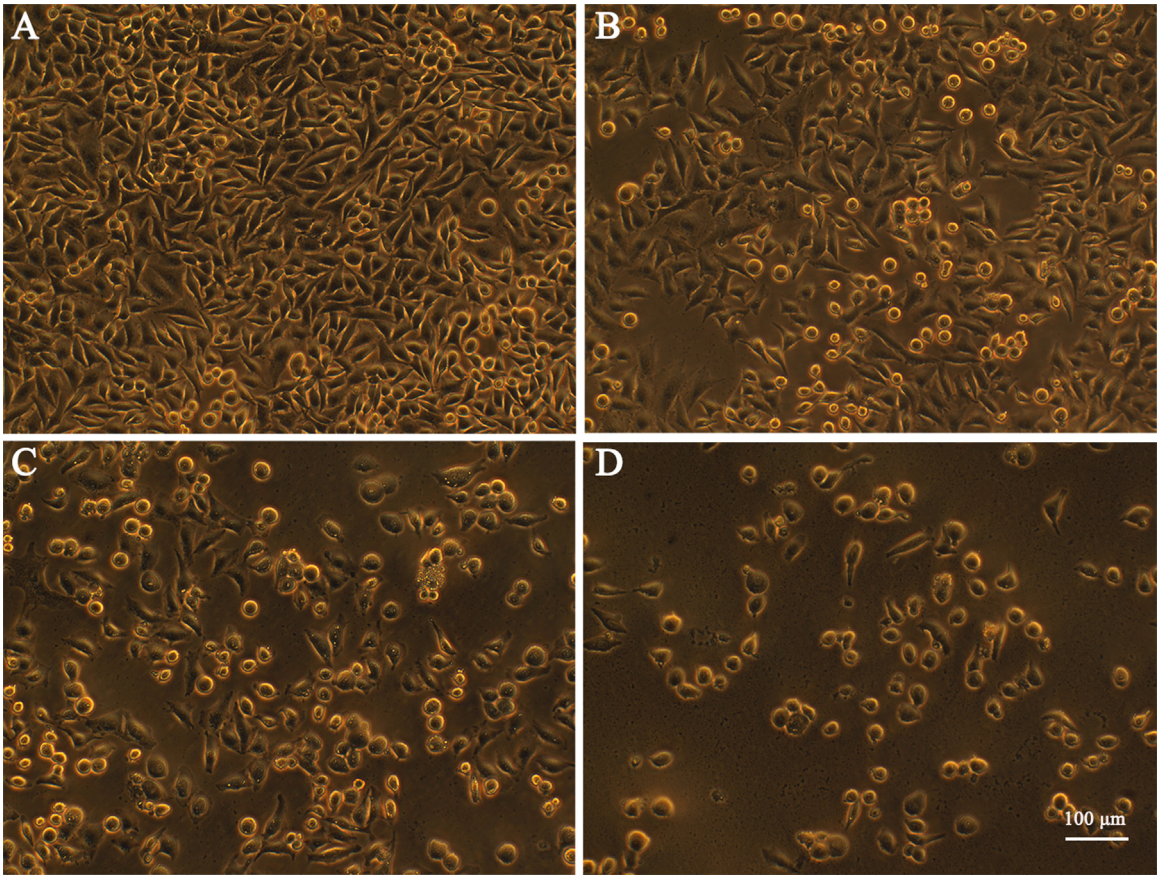


Fig. 2. Morphological observations of SGC-7901 cells treated for 48 h with the (A) control, (B) 200 μg/mL EEC, (C) 400 μg/mL EEC or (D) 800 μg/mL EEC. Scale bar = 100 μm.

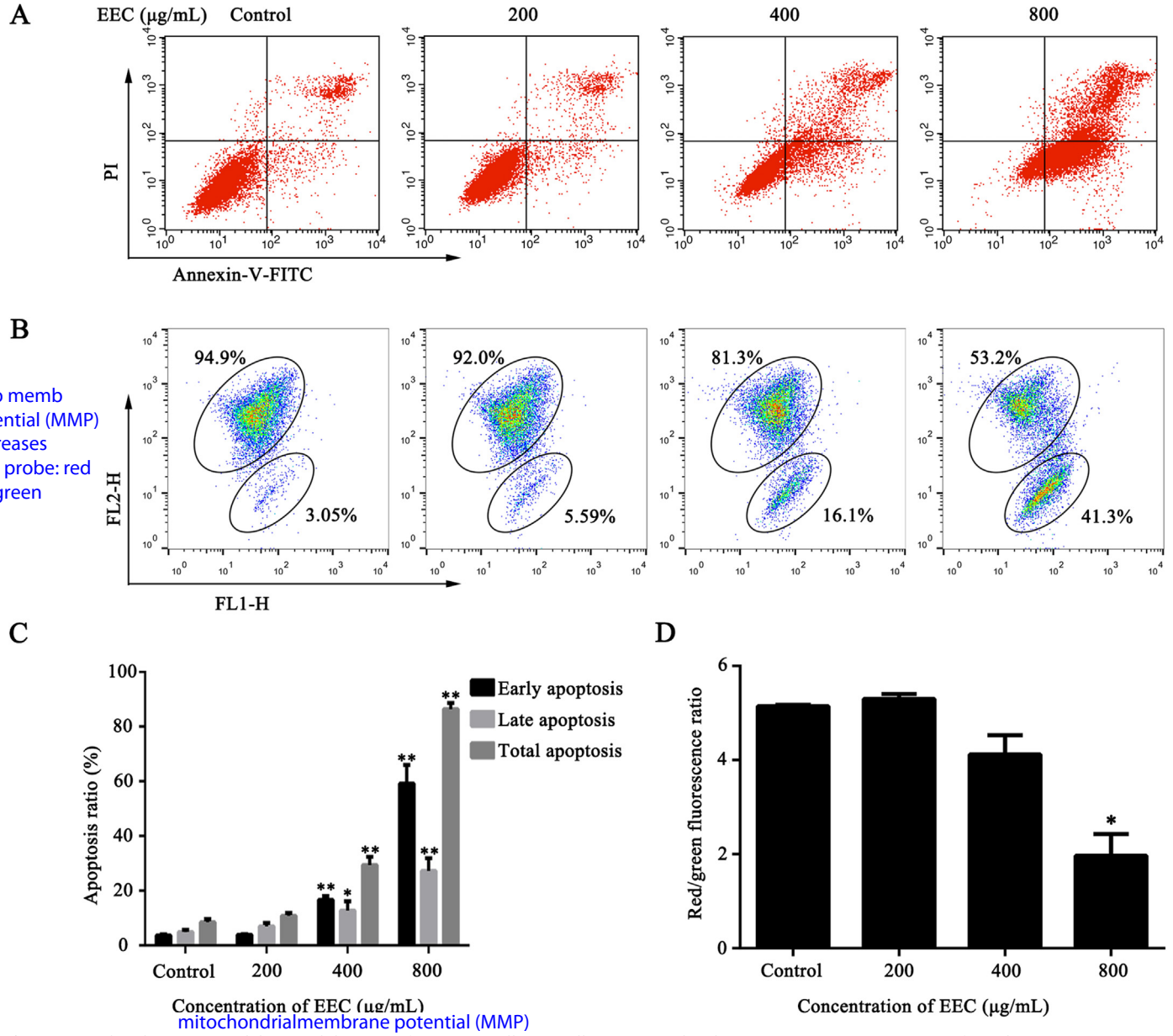


Fig. 3. EEC induced apoptosis and decreased the MMP of SGC-7901 cells. (A) Cells were treated with EEC (0, 200, 400 or 800 μg/mL) for 48 h. Apoptosis rates were quantified through the Annexin V-FITC/PI assay. (B) Cells were treated with EEC (0, 200, 400 or 800 μg/mL) for 48 h, and MMP was measured through JC-1 probe labelling. MMP in the treatment groups remarkably decreased from 94.9% to 53.2% relative to that in the control group. (C) After 48 h of treatment, the percentage of cells undergoing early apoptosis increased from 3.59% to 59.16% and that of cells undergoing late apoptosis increased from 4.88% to 27.24%. (D) Histograms of the average fluorescence intensity of MMP in SGC-7901 cells under EEC treatment. Data are shown with mean ± SD (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the control group.

The JC-1 probe can selectively enter the mitochondria and changes its colour from red to green as membrane potential decreases.

however, the number of adherent SGC-7901 cells decreased, and the cells became loosely attached, shrunken, rounded and randomly oriented (Fig. 2B–D). Treatment with high doses of EEC resulted in falling of cells from the cell plates (Fig. 2D). This result indicates that EEC induces the development of abnormal cell morphology in a dose-dependent manner.

3.2. EEC promoted the death receptor-induced apoptosis of SGC-7901 cells

EEC treatment exerted intense inhibitory effects on SGC-7901 cells. Thus, we determined apoptosis rates under EEC treatment through flow cytometry. The apoptosis rates of cells under treatment with increasing doses of EEC were higher than those of cells cultured under normal conditions (Fig. 3A, C). The percentage of cells undergoing early apoptosis increased from 3.59% to 59.16% and that of cells undergoing late apoptosis increased from 4.88% to 27.24%. These effects indicate that EEC induces apoptosis in a dose-dependent manner (Fig. 3C). Subsequently, we detected the expression of the key regulators of apoptosis through Western blot analysis after EEC treatment. The results of Western blot analysis indicate that the expression of the proapoptosis protein Bax was remarkably up-regulated, whereas that of the antiapoptosis regulator Bcl-2 was down-regulated (Fig. 4A, B). In addition, EEC treatment resulted in the up-regulated expression of caspase-3, caspase-6, caspase-8, AIF, p53 and Fas and the cleavage of PARP (Fig. 4A, B). These effects may promote apoptosis. These results suggest that EEC induces Fas, caspase-8, caspase-3 and caspase-6 expression and validate the occurrence of death receptor-induced extrinsic apoptosis under EEC treatment.

BAX: BCL2 Associated X, Apoptosis Regulator
Under stress conditions, undergoes a conformation change that causes translocation to the mitochondrion membrane, leading to the release of cytochrome c that then triggers apoptosis.

Bcl-2 family proteins contribute to programmed cell death or apoptosis.

外8内9-->3,7 -> 6

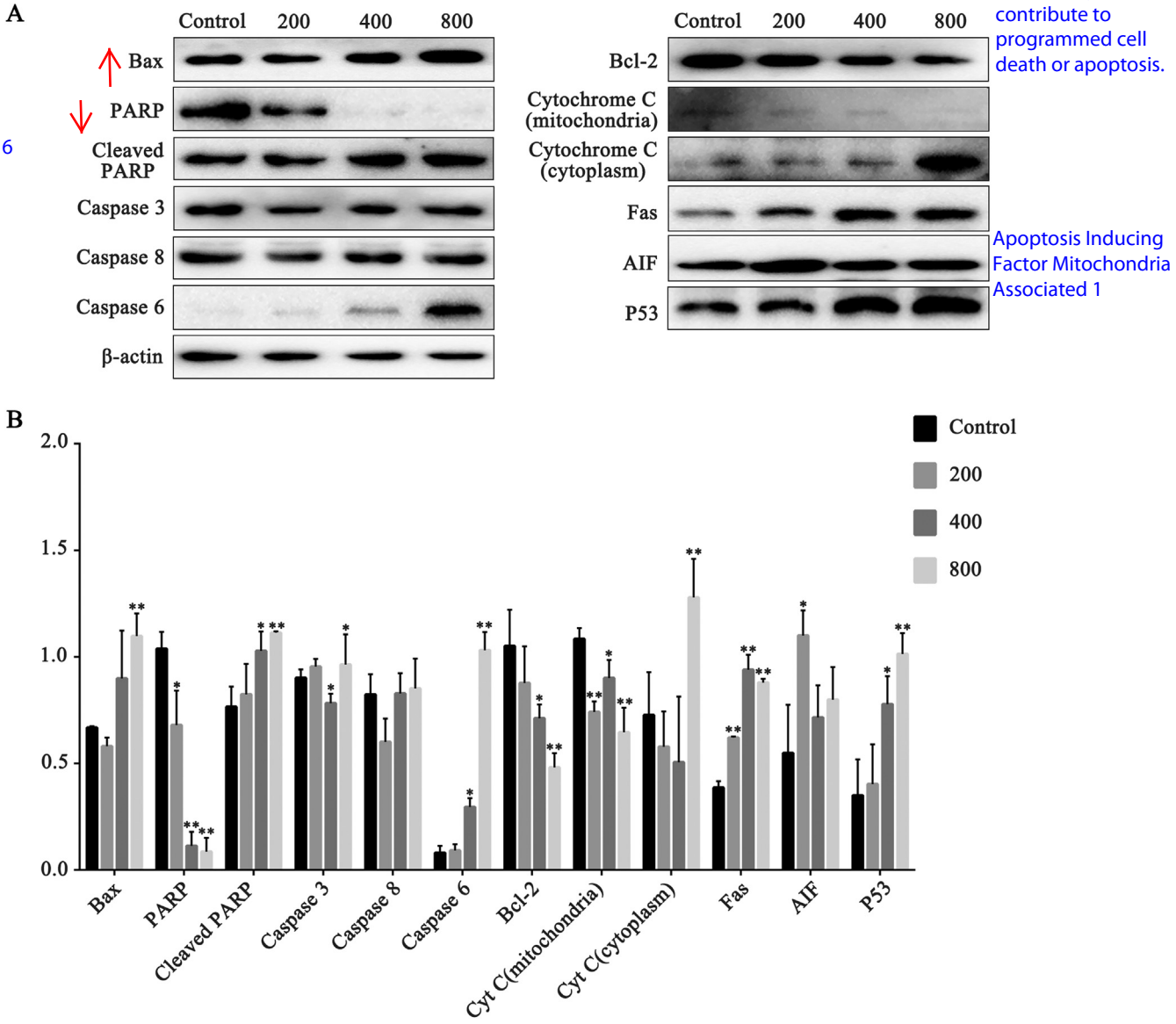


Fig. 4. Effect of EEC on proteins involved in the regulation of SGC-7901 cell apoptosis (A). Cells were treated with EEC (0, 200, 400 or 800 μg/mL) for 48 h, and proteins were extracted for Western blot analysis. (B) Histograms showing the expression levels of proteins involved in the regulation of apoptosis. The expression levels of target proteins were normalised to those of β-actin. Data are shown with mean ± SD (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the control group.

3.3. EEC decreased MMP and induced mitochondria-mediated apoptosis

Mitochondria play an important role in cell apoptosis. The JC-1 probe can selectively enter the mitochondria and changes its colour from red to green as membrane potential decreases. JC-1 staining was performed to determine the changes in MMP after EEC treatment to explain the function of mitochondria in EEC-induced apoptosis. Compared with that in the control treatment, MMP remarkably decreased under EEC treatment (94.9–92.0%, 81.3% and 53.2%) (Fig. 3B). Moreover, the value of MMP represented by Geo Means demonstrates that red fluorescence was remarkably attenuated, whereas green fluorescence intensified. These results imply that EEC can decrease MMP in a dose-dependent manner (Fig. 3D). Western blot analysis was performed to quantify the levels of apoptosis-associated proteins after changes in MMP. The expression levels of cytochrome c, caspase-3 and caspase-6 were elevated. Therefore, the above data suggest that EEC treatment decreases transmembrane potential by increasing membrane permeability. This effect is followed by the release of cytochrome c from the mitochondria to the cytoplasm. These phenomena ultimately result in apoptosis due to the activation of downstream apoptosis-related factors, such as caspase-3 and caspase-6 (Fig. 4A, B).

3.4. EEC arrested SGC-7901 cells in the S phase

Given the observation that EEC suppressed the proliferation of SGC-7901 cells, the association between EEC-induced cell death and cell cycle arrest was examined. After EEC treatment, the number of cells in the S phase remarkably increased from 36.06% to 72.93%, whereas that in the G1 phase decreased from 55.95% to 20.16% and that in the G2 phase decreased from 8.0% to 6.91% (Fig. 5A, C). The expression of factors involved in controlling cell cycle progression was investigated to identify the mechanisms underlying EEC-induced S-phase arrest. The results indicate that Rb underwent phosphorylation under EEC treatment. Rb phosphorylation (P-Rb), in turn, induced the expression of the transcription factor E2F and promoted the transcription of cyclin E and Cyclin A. These results imply that the overexpression of cyclin E/CDK2 and cyclin A/CDK2 has a key role in EEC-induced S phase arrest

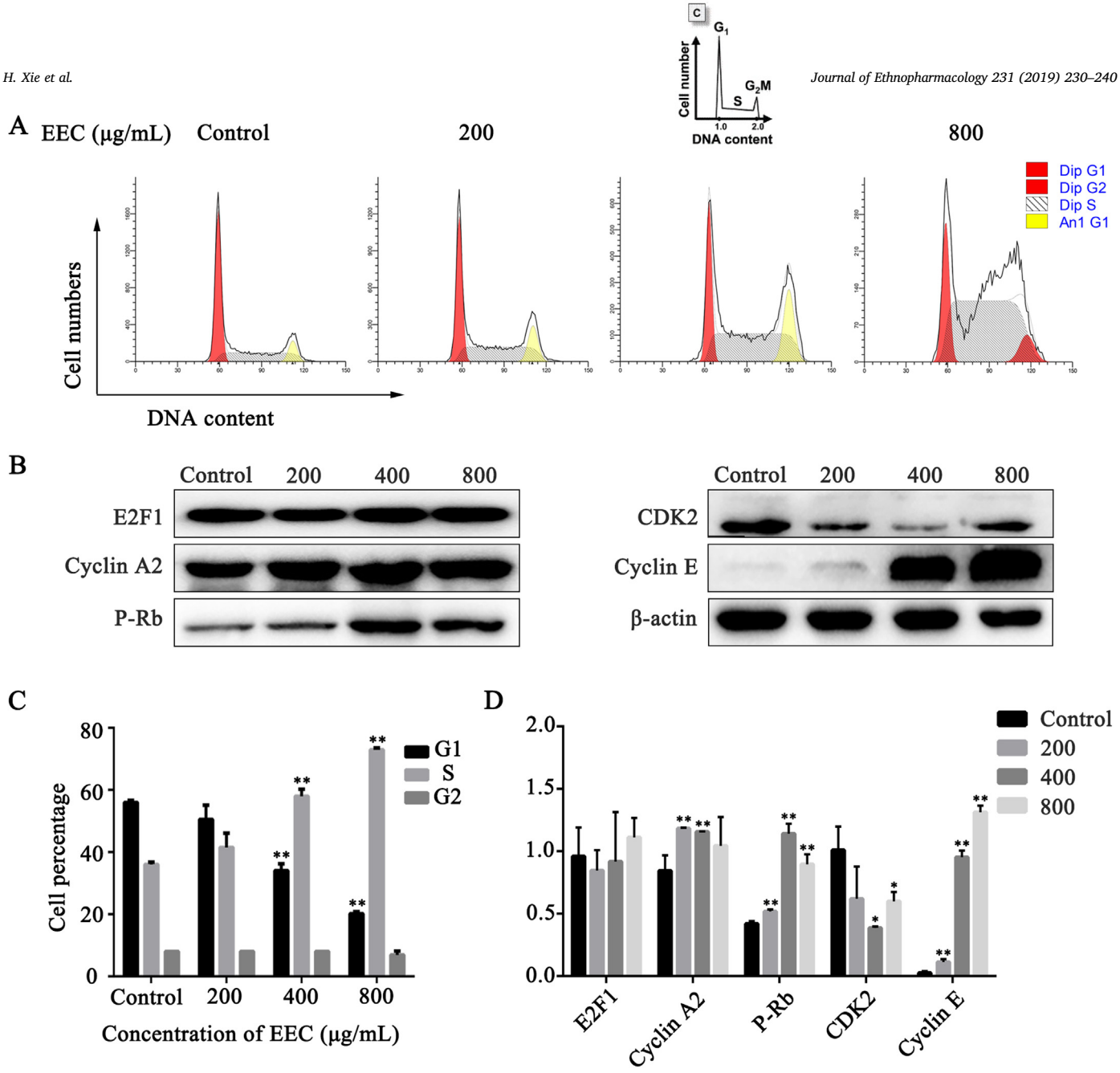
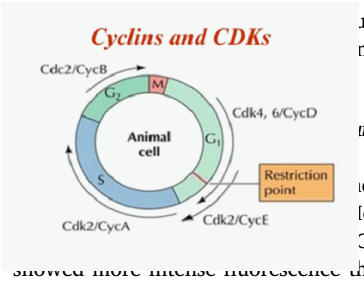
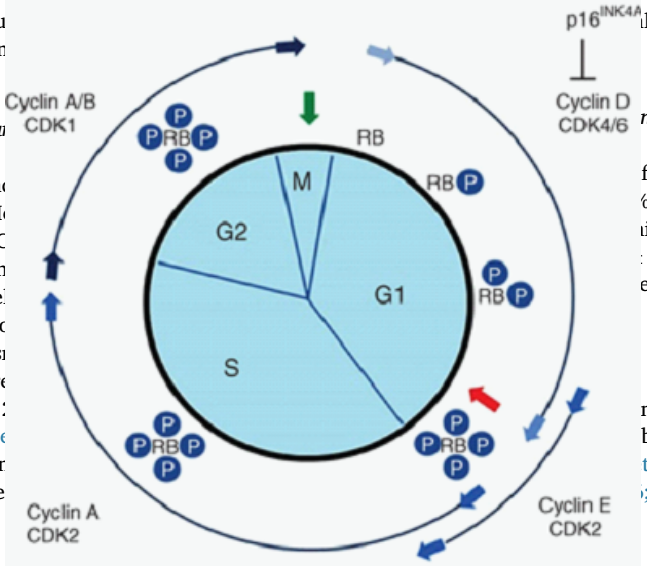


Fig. 5. EEC treatment arrested SGC-7901 cells in the S phase. (A) PI-stained SGC-7901 cells subjected to flow cytometry for the detection of cell cycle distribution. (B) Western blot analysis of proteins related to S phase arrest under EEC treatment. Cells were treated with EEC (0, 200, 400 or 800 μg/mL) for 48 h, and the extracted proteins were subjected to Western blot analysis. (C) Quantisation of cell cycle distribution. (D) Histograms of the expression levels of cell-cycle regulatory proteins quantified through Western blotting analysis. Data are shown as mean ± SD (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the control group.





lly involved in the antitumor effect of EEC

nent analysis of EEC

furic acid assay show that the total sugar % (w/w). The results of the HPLC assay ined 1.841 ± 0.007, 6.015 ± 0.018, 0.019 μg/mg adenine, uridine, adenosine enosine, respectively (Table S1).

ment (Fig. 6A). The increase in the re 12.50 to 44.56 suggests that Ca^{2+} c treatment (Fig. 6A, C). The endoplas site of intracellular Ca^{2+}. During stre doplasmic reticulum and caspase-1 ticulum stress, is activated (Banerjee 1, caspase-12 and caspase-9 protein treated groups. This finding indicate

medicine, the therapeutic and health care been previously validated. These functions t al., 2016; Sharma et al., 2015), anti- ; Sangdee et al., 2015), anti-inflammatory

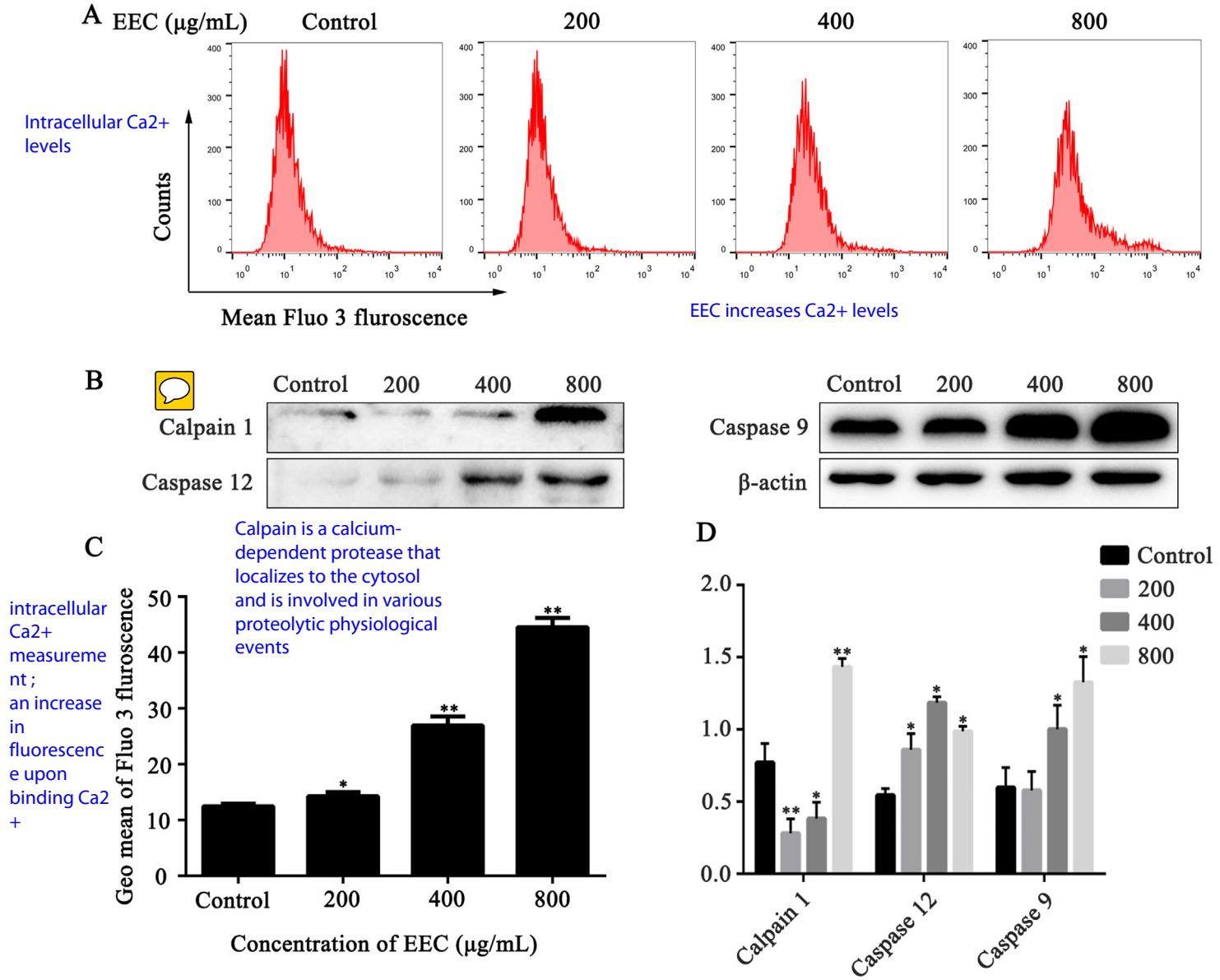


Fig. 6. EEC induced endoplasmic reticulum stress in SGC-7901 cells. (A) Intracellular Ca^{2+} levels in SGC-7901 cells were determined through flow cytometry with Fluo-3 AM fluorescent dye after 48 h of treatment with EEC (0, 200, 400 of 800 μg/mL). (B) Effect of EEC on the expression of proteins related to endoplasmic reticulum stress. (C) Histograms of the average fluorescence intensity of Fluo-3 AM probes in SGC-7901 cells under EEC treatment. (D) Histograms showing the expression levels of proteins related to endoplasmic reticulum stress. Data are shown as mean ± SD (n = 3). $^{**}p < 0.01$, $^{*}p < 0.05$ compared with the control group.

(Lu et al., 2015), renoprotective (Zhu et al., 2014), immunomodulatory (Yang and Zhang, 2016) and anticancer effects. *C. cicadae* is a functionally safe food (Chen et al., 2015). In this work, we investigated the antitumor mechanism of EEC. We performed the MTT assay to examine the cytotoxicity of EEC against different types of cancer cells after 48 h of treatment. Amongst all of the cancer cell lines tested in this work, SGC-7901 cells showed the highest sensitivity to EEC (Fig. 1). The results for the morphological assay of SGC-7901 cells show that EEC induces morphological changes in a concentration-dependent manner (Fig. 2). Subsequent investigations have proven that EEC exerts its antitumor effect by promoting cell apoptosis (Fig. 3A), inducing cell cycle arrest in the S phase (Fig. 5A) and increasing endoplasmic reticulum stress. In addition, the results of our immunoblot assay have revealed that specific cell death signalling pathways are activated in response to EEC treatment. Polysaccharides and nucleosides, the main components of *Cordyceps*, exert antitumor effects (Cai et al., 2018). We identified adenine, uridine, adenosine and N6-(2-Hydroxyethyl)-adenosine as the main active components of EEC (Fig. 7). Adenosine and N6-(2-Hydroxyethyl)-adenosine are the main active components of *C. cicadae*, and uridine and adenine are also found in natural *Cordyceps* (Zhao et al., 2013; Lu et al., 2015).

Apoptosis and endoplasmic reticulum stress are the two key determinants of cell death. In mammalian cells, caspase-dependent apoptosis occurs through the two distinct molecular pathways of death receptor-induced extrinsic apoptosis and mitochondria-mediated intrinsic apoptosis (Ashkenazi and Dixit, 1998; Budihardjo et al., 1999; Kiraz et al., 2016; McIlwain et al., 2015; Shi, 2002). Our results indicate that EEC increases Fas, caspase-8, caspase-3 and caspase-6 levels and suggest that the extrinsic apoptosis pathway is induced in SGC-7901 cells (Jin and El-Deiry, 2005). The results of MMP and Western blot analysis confirm our speculation that the intrinsic apoptosis pathway is activated after EEC treatment. In addition, Ca^{2+} overload causes endoplasmic reticulum stress (Szegezdi et al., 2003; Timmins et al., 2009). Here, we discovered that EEC increases Ca^{2+} levels in SGC-7901 cells

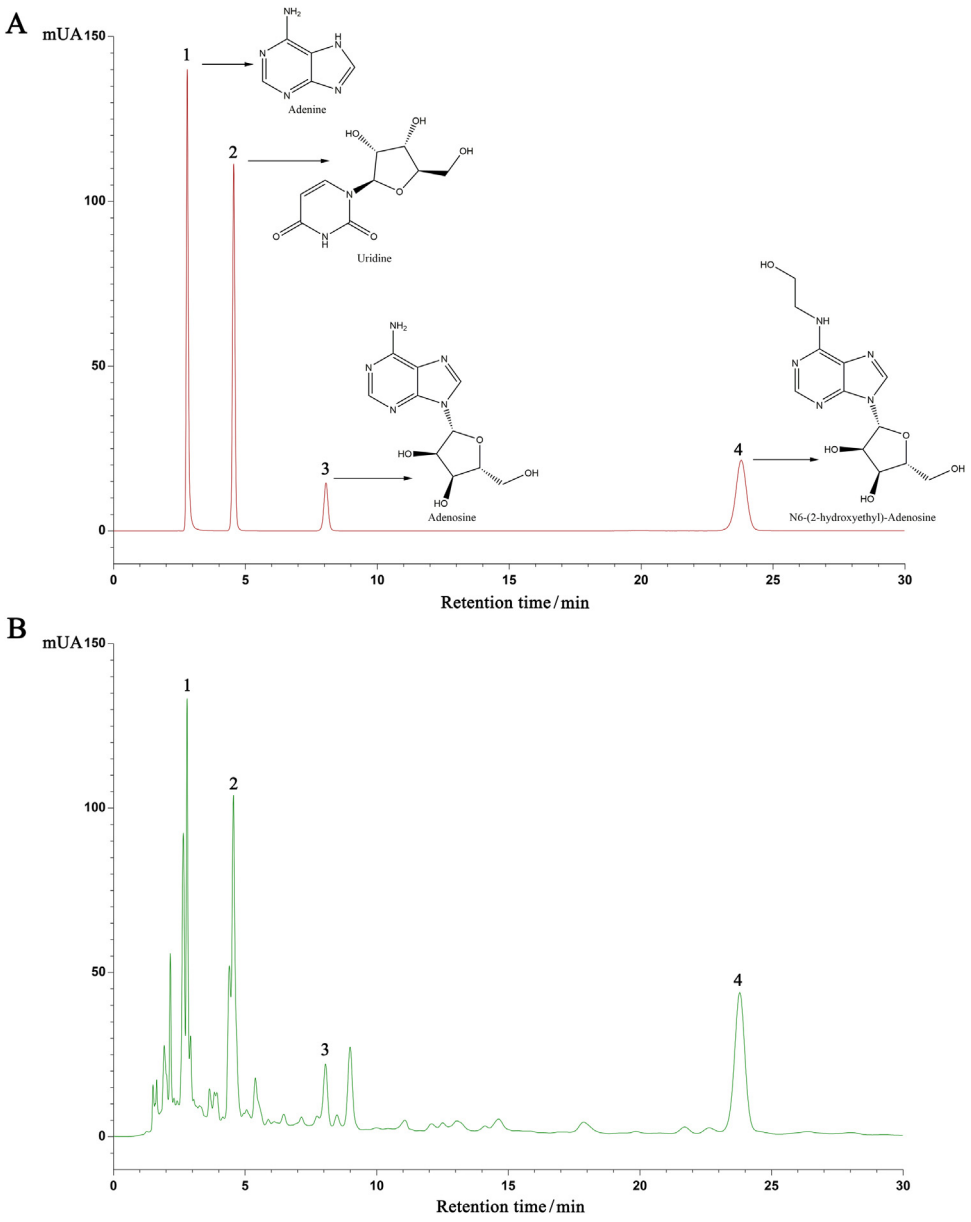


Fig. 7. HPLC of mixed standards and EEC. (A) Mixed standards (adenine, uridine, adenosine and N6-(2-Hydroxyethyl)-adenosine). (B) EEC (30 mg/mL). (1) Adenine, (2) uridine, (3) adenosine and (4) N6-(2-Hydroxyethyl)-adenosine.

(Fig. 6A). The results of Western blot analysis indicate that the levels of calpain-1, caspase-12 and caspase-9 proteins have increased after EEC treatment (Fig. 6B). Prolonged endoplasmic reticulum stress causes apoptosis and/or autophagy and cell death (Hetz, 2012; Momoi, 2004). Therefore, the signalling pathway of endoplasmic reticulum stress may be involved in SGC-7901 cell death under EEC treatment.

The results of our cell cycle assay suggest that EEC induces cell cycle arrest in the S phase (Fig. 5A). Cyclin proteins interact with their corresponding cyclin-dependent kinases (CDKs) to form cyclin/CDK complexes that regulate cell cycle processes. The up-regulation of cyclin E/CDK2 and cyclin A/CDK2 formation is involved in the mechanism of S phase arrest (Fig. 5B). P53 participates in apoptosis, genomic stability and angiogenesis inhibition, can inhibit growth by arresting the cell cycle at the G1/S regulation point on DNA damage identification (Arima et al., 2004; Futamura et al., 2007). Activated p53 promotes p21 expression, inhibits CDK2 activity and induces cell cycle arrest (Schwartz and Shah, 2005). Rb and transcription factor E2F are the cyclin/CDK substrates that regulate G1/S phase transition (Nevins, 2001). Cyclin A is required for DNA replication, and the cyclin E/CDK2 complex is the key kinase complex that modulates the transition from G1 phase to S phase (Fig. 8) (Dulic et al., 1992; Girard et al., 1991).

Antitumor therapy could elicit curative responses through different signalling pathways leading to cell death (Portugal et al., 2009). The mechanism of action of chemotherapeutic agents involves apoptosis activation and cell cycle blockage (Rowinsky, 2005; Stewart et al., 2003). Antitumor therapy causes apoptosis and cell cycle arrest and promotes cancer cell death through different avenues, such as necrosis (Ricci and Zong, 2006), endoplasmic reticulum stress (Boelens et al., 2007), senescence (Roninson, 2003) and autophagy (Zhong et al., 2016). In this work, we postulated that the antitumor activity of EEC involves multiple processes, such as the activation of apoptosis, blockage of the cell cycle at the S phase and induction of endoplasmic reticulum stress. Antroquinonol, which is isolated from *Antrodia camphorate*, triggers mitochondria-dependent apoptosis, senescence and autophagy in human pancreatic cancer cells (Yu et al., 2012). A ribonuclease isolated from wild *Ganoderma lucidum* induces apoptosis in addition to G1 phase arrest in colorectal cancer cells (Dan et al., 2016). The β-glucan extract of *Lentinus edodes* inhibits the proliferation of

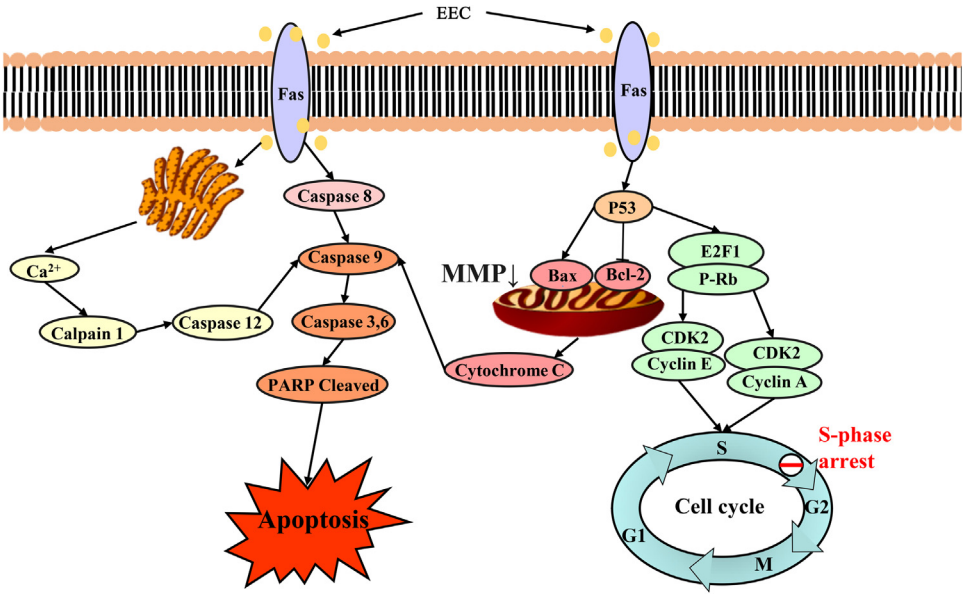


Fig. 8. Schematic of the pathway underlying the cytotoxic effect of EEC on SGC-7901 cells. EEC causes cell apoptosis by enhancing p53 and Bax expression, inhibiting Bcl-2 expression, decreasing MMP, promoting cytochrome C release, activating downstream caspase pathways and cleaving PARP in the nucleus. EEC enhances p53 expression and Rb phosphorylation. The combination of phosphorylated Rb with E2F1 increases cyclin E/CDK2 and cyclin A/CDK2 levels and results in S phase arrest. EEC induces endoplasmic reticulum stress through Ca^{2+} overloading, increasing calpain-1 and caspase-12 expression and activating downstream caspase signalling pathways.

breast carcinoma cells by promoting apoptosis and cell cycle arrest at the G2/M phase (Xu et al., 2017). *C. militaris* induces the apoptosis, cell cycle arrest and autophagy of human glioblastoma cells (Yang et al., 2012). Consistent with previous studies, the present study shows that natural bioactive products separated from fungi exhibit antineoplastic activity through single and multiple processes. Future studies should investigate whether the antitumor effects of *C. cicadae* involve other mechanisms, such as autophagy and necrosis.

Most chemotherapeutic agents are assumed to induce cancer cell death directly through apoptosis, autophagy or necrosis. Moreover, considerable evidence has shown that activated immune cells may exert antitumor effects. Immune responses to tumours are mediated by antigen-specific and nonspecific mechanisms that are produced by immune cells and their secreted soluble factors, such as inflammatory cytokines and chemokines (D'Elios et al., 2009). Chitosan isolated from seafood enhances the antineoplasmic activity of natural killer cells by activating dendritic cells (X.X. Li et al., 2018). Bleomycin exerts a contradictory antineoplastic immune effect by causing immunogenic cell death and regulatory T cell proliferation (Bugaut et al., 2013). In murine bone marrow-derived myeloid dendritic cells, *C. militaris* promotes the phenotypic and functional maturation of dendritic cells and increases the expression levels of CD40, CD54, CD80, CD86 and MHC class II (Kim et al., 2006). The *in vitro* and *in vivo* antitumor and antimetastatic activities of *C. taii* have been validated, and *C. taii* polysaccharides present antioxidant activities that can enhance immune function in a mouse model of D-galactose-induced aging (Liu et al., 2015; Xiao et al., 2012b). *C. cicadae* plays an immunoregulatory role because it promotes the growth of human mononuclear cells and activation of T lymphocytes (Kuo et al., 2003; Weng et al., 2002). Future studies should investigate whether the antitumor activity of EEC includes its immunoregulatory effects.

In conclusion, we found that EEC suppresses the growth of SGC-7901 cells by inducing apoptosis, cell cycle arrest and endoplasmic reticulum stress (Fig. 8). Mechanism experiments showed that EEC treatment activates death receptor-induced extrinsic and mitochondria-mediated intrinsic apoptosis pathways, which are involved in caspase-dependent apoptosis. EEC treatment arrested SGC-7901 cells in the S phase under the regulation of cyclins and CDKs. Our results collectively indicate that *C. cicadae* is a promising chemopreventive and chemotherapeutic agent for gastric cancer.

CRediT authorship contribution statement

Hongqing Xie: Methodology, Data curation, Investigation, Writing-original draft. **Xiaotong Li:** Methodology, Data curation, Writing-review & editing. **Yajie Chen:** Methodology, Data curation, Investigation, Writing-review & editing. **Mingzi Lang:** Methodology. **Zhangfei Shen:** Methodology, Investigation, Writing-review & editing. **Liangen Shi:** Methodology, Investigation, Resources, Writing-review & editing.

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Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.11.028.

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