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Polyphenols Extracted from *Enteromorpha clathrata* Induce Apoptosis in Hepa1-6 Cell by Activating the Mitochondrial Apoptosis Signaling Pathways

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Abstract Green alga *Enteromorpha clathrata* (*E. clathrata*) contains a variety of bioactive compounds, including polysaccharides, polyphenols and fat-soluble pigments etc., among which polyphenols exhibit a wide range of medicinal properties. *E. clathrata* polyphenols (ECPs) have shown various biological activities such as antioxidant, anti-inflammatory and antidiabetic effects; however, the potential of ECPs as an anti-cancer reagent remains unclear. The aim of this study was to investigate the anti-tumor activity and underlying mechanisms of ECPs on hepatocellular carcinoma. The cytotoxicity of Hepa1-6 cells was determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assay. Flow cytometry and fluorescence microscope analysis of cell apoptosis after annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay was used for intracellular reactive oxygen species (ROS) detection. caspase-9 activity was determined using cspase-9 colorimetric assay. Mitochondrial transmembrane potential ($\Delta\psi m$) was measured using JC-1. Western blot and quantitative real-time PCR (qPCR) were used to assess the expressions of the apoptosis regulators Bax, Bcl-2, cytochrome c and caspase-3. It was found that ECPs showed a dose-dependent cytotoxicity against Hepa1-6 cells by inducing apoptosis. The apoptosis in ECPs-treated Hepa1-6 cells was accompanied by the loss of mitochondrial membrane potential, elevated ROS generation, increased release of mitochondrial cytochrome c, and up-regulation of caspase-9 and caspase-3. The expressions of Bax (pro-apoptotic molecule) and Bcl-2 (apoptosis suppressor) were up-regulated and down-regulated, respectively, at both mRNA and protein levels. These molecular alterations revealed that ECPs caused apoptosis of cells through the mitochondrial pathway, suggesting that ECPs are potential candidates to be developed for liver cancer treatment.

Key words *Enteromorpha clathrate*; polyphenol; anti-cancer activity; apoptosis; mitochondrial-dependent pathway

1 Introduction

Liver cancer is one of the most common malignant tumors with high morbidity and mortality. The treatment of liver cancer remains a challenge due to the complexity of tumour pathology and the limitations of current methods (surgery, radiotherapy, and cytotoxic chemotherapy) (Mohammad *et al*., 2015; Wang *et al.*, 2015). Conventional cytotoxic chemotherapy drugs are primarily designed to destroy the rapidly proliferating cancer cells (Murphy *et al*., 2014). Unfortunately, many healthy cells, especially those with high proliferation rate like cells in the bone marrow, intestinal villi and hair follicles, can also be damaged, resulting in moderate to severe side effects including nausea, anaemia, impaired immunity, hair loss, vomiting and diarrhoea (Kintzios *et al*., 2004; Vanneman *et al*., 2012). Moreover, resistance to conventional chemotherapeutic drugs due to the heterogeneous nature of tumours and their genetic mutations, is also one of the major challenges in cancer therapy (Dropcho, 2011; Sundarraj *et al*., 2020). Recently, increasing research has focused on developing cytotoxic drugs with high specificity to tumour cells to precisely inhibit or block their growth and proliferation (Vidya *et al*., 2012; Hu *et al*., 2018). However, to date, most novel reagents still require to work together with classical chemotherapeutic drugs, radiotherapy or surgery. One approach to develop new potential cytotoxic chemotherapeutic reagents is to identify bioactive natural products with anti-tumour activity.

Macroalgae, commonly known as seaweeds, have been consumed in Asia for centuries, while Chinese is the biggest consumer (Paiva *et al*., 2016). The low prevalence of diet-related cancers in areas with high algae consumption has been demonstrated in epidemiological studies, indicat-

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ing the potential of algae as a source of anti-cancer reagents (Yuan *et al*., 2007; Teas *et al*., 2011). The presence of cytotoxic substances in seaweeds is not surprising since their compounds (polysaccharides, polyphenols and carotenoids, *etc*.) can protect against herbivory and encroachment of other marine organisms into their habitat (Fleurence, 1999; Paiva *et al*., 2016). During the past decades, the anticancer studies of seaweed mainly focus on brown algae, and most of them are crude extracts and polysaccharides (Murphy *et al*., 2014). In recent years, there have been increasing reports on antitumor activity of seaweed derived polyphenols. For instance, crude polyphenol extracted from *Ecklonia cava* (brown algae) and *Eucheuma cottonii* (red algae) can induce apoptosis of colon cancer cells and breast cancer cells, respectively (Athukorala *et al*., 2006; Yuan *et al*., 2012). *Enteromorpha clathrata* (Chlorophyta, Ulvaceae), an edible seaweed with high nutritional and medicinal value (Yuan *et al*., 2012; Sun *et al*., 2017), is popular in coastal areas of Asia, such as China and Japan. However, it is one of the main species that trigger 'green tides' threatening aquatic ecosystem (Zhong *et al*., 2020). Thus, the exploitation and utilization of *E. clathrata* have been considered as a good strategy for both environment protection and natural resource application. There has been research demonstrating the antitumor activity of *Enteromorpha* and its constituents. For instance, the extract of *E. prolifera* with methanol/acetone can effectively reduce the occurrence of skin tumors in mice (Hiqashi-Okaj *et al*., 1999). Sulfated polysaccharides isolated from *E. prolifera* appeared to inhibit the growth of murine gastric adenocarcinoma cancer cells and human colon adenocarcinoma cancer cells (Cho *et al*., 2010). Methanol extract of *E. clathrata* was also found to suppress the growth of transformed mouse 3T3 cells (Tang *et al*., 2004). At present, studies have demonstrated that the *E. clathrata* polyphenols (ECPs) possess various biological functions including antioxidant (Wang *et al*., 2021), cholesterol-lowering (Feng *et al*., 2016) and anti-inflammatory activities (Huang *et al*., 2022). However, to the date, the potential of polyphenols derived from *E. clathrata* in the chemoprevention and treatment of liver cancer remains unclear.

The loss of apoptotic control is closely related to the initiation and progression of liver cancer (Alem *et al*., 2019). Thus, induction of cancer cell apoptosis has been recognized as an important method in cancer therapy. The objective of the present research was to explore the anti-cancer activity of *E. clathrata* polyphenols (ECPs) against mouse hepatocarcinoma cells, with specific focus upon the intrinsic mitochondrial pathway of apoptosis−the most commonly deregulated form of cell death in cancer.

2 Materials and Methods

2.1 Materials

Hepa1-6 cells (Procell CL-0105) were provided by Procell Life Science and Technology Co., Ltd. Dulbeccos modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin/streptomycin solutions were obtained from Corning (NY, USA), Lactate dehydrogenase (LDH) was purchased from Beyotime. Dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl-)-2, 5-diphenyl tetrazolium bromide (MTT), assay kits for reactive oxygen species (ROS) detection assay kit, annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit, caspase-9 assay kit (colorimetric), bicinchoninic acid (BCA), phenylmethylsulfonyl fluoride (PMSF), radio immunoprecipitation assay (RIPA) lysis buffer (high), protein loading buffer 4×, JC-1 mitochondrial membrane sensor kit, tris-buffered saline and tween 20 (TBST) were purchased from Beijing Solarbio Science & Technology Co., Ltd. Goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) and protease inhibitor were provided by Beijing ComWin Biotech Co., Ltd. Skim milk powder was from BBI. Taq-based PCR enzyme was supplied by Toyobo (Osaka, Japan). Monoclonal antibodies for Bax, Bcl-2, cytochrome c and active+pro caspase-3 were products of ABclonal Technology Co., Ltd. NcmECL Ultra was purchased from New Cell and Molecular Biotech Co., Ltd.

2.2 Isolation and Separation of Polyphenols from *E. clathrata*

The dry *E. clathrata* was obtained from Lulin seafood market (Ningbo, Zhejiang). The species verification was conducted by Dr. Jinjie Zhang (Ningbo University). Ultrasound-assisted extraction approach was applied to obtain different organic fractions from *E. clathrata* using ethanol and three organic solvents (petroleum ether, ethyl acetate, n-butanol) sequentially. The total polyphenol content of the extract (*i.e*., ECPs) was 32.23 mg g^{-1} (Wang *et al*., 2021). The ECPs, identified as fraction with potent biological activity (Wang *et al*., 2021; Huang *et al*., 2022), was selected for evaluating its anti-cancer property.

2.3 Cell Culture

Hepa1-6 cells were cultured in DMEM supplemented with 10% FBS, 1% sodium pyruvate and 1% penicillin/streptomycin. They were maintained at 37℃ in a 5% CO_2 humidified incubator and were passaged at 80%−90% confluence.

2.4 Cell Viability

Cell viability was determined using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Hepa1-6 cells were seeded in 96-well plates (3×10^4 cells per well) prior to treating them with ECPs at 0−0.32 mg mL^{-1} dose for 24 h or 48 h. After exposure, the supernatant was replaced with MTT solution (0.5 mg mL^{-1}) and further incubated for 4 h. Afterwards, MTT was discarded and replaced with 150 μL of dimethyl sulfoxide (DMSO) to solubilize the formazan crystals. The absorbance was measured at 540 nm using SpectraMax i3 Platform (Molecular Devices, USA). Cell viability was expressed as a percentage relative to the control group.

The half maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 8 (GraphPad, San Diego, CA, USA) according to the relative cell viability.

2.5 Lactate Dehydrogenase (LDH) Cytotoxicity Assay

The cytotoxicity of ECPs towards Hepa1-6 cells was measured using LDH cytotoxicity assay kit according to the manufacturer's instructions. Briefly, Hepa1-6 cells were seeded in 96-well plates at a density of 3×10^4 cells per well and incubated for 10 h, followed by exposure to ECPs at 0–0.32 mg mL^{-1} dose for 24 h or 48 h. Non-exposed cells were lysed to obtain the maximum LDH release. The supernatant of each well was collected and incubated with LDH working solution for 30 min in the dark. The absorbance was measured at 490 nm with SpectraMax i3 Platform (Molecular Devices, USA). The cytotoxicity was expressed as the percentage of LDH release (%) relative to the control group.

2.6 Flow Cytometry and Fluorescence Microscope Analysis of Cell Apoptosis

Cell apoptosis was evaluated by flow cytometry using Annexin V-FITC/PI double staining assay according to the manufacturer's instructions. Hepa1-6 cells were seeded in 6-well plates at a density of 1×10^6 cells per well, and treated with ECPs (0.1 and 0.2 mg mL^{-1}) for 24 h. Cells were collected, centrifuged, and resuspended in 1 mL of 1× binding buffer. 5 μL of Annexin V-FITC solution was added to the cell suspension, and incubated for 10 min at room temperature in the dark. 5 μL of PI solution was added to the cells, followed by an additional 5 min incubation. The scatter parameters of the cells were analyzed by flow cytometry and data were processed with Flow Jo software vX.0.7 (Tree Star, USA). Four cell populations were identified, including viable population (low-PI and FITC signals; lower-left quadrant), early apoptotic population (low-PI and high-FITC signals; lower-right quadrant), necrotic population (high-PI and low-FITC signals; upper-left quadrant), and late apoptotic or necrotic population (high-PI and high-FITC signals; upper-right quadrant).

Cell apoptosis was also examined using fluorescence microscope (Nikon ECLIPSE Ts2R-FL, Japan). Cells were seeded in 6-well plates (1×10^6 cell per well) and treated by ECPs (0.1 and 0.2 mg mL^{-1}) for 24 h. Cells were washed with PBS and 1× binding buffer, followed by Annexin V-FITC/PI double staining in the manner described above. Cells were then subjected to fluorescence microscope analysis with 488 nm excitation and 525 nm (FITC) or 620 nm (PI) emission wavelengths. Bright green fluorescence was manifested in membranes of the cells undergoing prophase apoptosis (Annexin V-FITC staining), and nuclear cardinal red fluorescence was associated with apoptosis at further stages (PI staining).

2.7 Reactive Oxygen Species (ROS)

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay was used for intracellular ROS detection. After cell uptake, DCFH-DA is deacetylated by esterase to a non-fluorescent compound that is later oxidized by ROS into fluorescent 2',7'-dichlorofluorescin (DCF) which can be detected by fluorescence microscopy. In brief, cells were seeded in 24-well plates at a density of 2×10^5 cells per well, and exposed to ECPs (0.1 and 0.2 mg mL^{-1}) for 24 h. 400 μL of 10 μmol L^{-1} DCFH solution was added to each well and incubated for 30 min at 37℃. After being washed twice with PBS, 500 μL of serum-free medium was added each well. Intracellular ROS level was evaluated by determining the fluorescence intensity using SpectraMax i3 Platform (Molecular Devices, USA) with 485-nm excitation and 530-nm emission wavelengths.

2.8 Mitochondrial Membrane Potential (MMP)

The MMP of Hepa1-6 cells post ECPs treatment was measured using the JC-1 MMP assay kit according to the manufacturer's instructions. Briefly, Hepa1-6 cells (1×10^6 cells per well, 6-well plate) were exposed to ECPs for 24 h. Cells were collected and treated with JC-1, incubated at 37℃ for 20 min and then washed twice. Then they were observed under fluorescence microscope (Nikon ECLIPSE Ts2R-FL, Japan). The level of MMP was evaluated by determining the fluorescence intensity using Fluorospectrophotometer (F4700, Japan) with 490/525-nm excitation and 530/590-nm emission wavelengths and was expressed as the relative ratio of red (J-aggregates) fluorescence and green (monomer) fluorescence.

2.9 Caspase-9 Activity Assay

Caspase-9 activity was determined using caspase-9 colorimetric assay kit according to the manufacturer's instructions. Hepa1-6 cells (1×10^6 cells per well, 6-well plate) were treated with ECPs for 24 h. Cells were lysed with lysis buffer on ice for 15 min and the supernatant was obtained by centrifugation. The absorbance was determined at 405 nm using SpectraMax i3 Platform (Molecular Devices, USA).

2.10 Gene Expression Analysis

Hepa1-6 cells (1×10^6 cells per well, 6-well plate) were treated with ECPs for 24 h. Total cellular RNA was extracted with trizol reagent, and reversely transcribed into cDNA using the Taq-based polymerase chain reaction (PCR) enzyme kit in accordance with the manufacturer's instructions. The cDNA was applied to the quantitative real-time PCR (qPCR) using the FastStart Essential DNA Green Master and the validated primers. The sequences of the primers used for amplification of *Bcl-2*, *Bax* and *hprt1* transcripts were as follows: *Bax* forward, 5'-GA TCGAGCAGGGCG AATG-3' and reverse, 5'-TGAGGAGTCTCACCCAACC A-3'; *Bcl-2* forward, 5'-GGGAGAACAGGGTACGATAA-3' and reverse, 5'-CCCACCGAACTCAAAGAA-3'; *hprt 1* forward 5'-TCAGTCAACGGGGGACATAAA-3' and reverse, 5'-GGGGCTGTACTGCTTAACCAG-3'. *hprt 1* was used as the endogenous control. qPCR was performed using a CFX 96 Touch Real-time fluorescence quantitative qPCR instrument (Bio-Rad, USA) according to the following conditions: 95℃ for 10 min, followed by 39 cycles of 95℃ for 10 s, 60℃ for 15 s and 72℃ for 20 s. Data was analyzed using the $2^{-\Delta\Delta CT}$ method. by normalizing relative quantitation (RQ) values for experimental group to the control group.

2.11 Western Blot Analysis

Hepa1-6 cells were treated with ECPs as described above, and were collected and lysed with RIPA buffer. The total protein concentration of cell lysate was measured using BCA kit. The proteins in cell lysates were resolved on SDS-PAGE by electrophoresis, and then transferred to PVDF membranes for western blot analysis. Briefly, after being blocked in 5% non-fat milk, the membranes were probed with diluted primary antibodies (Bax, Bcl-2, caspase-3, cytochrome c and β-actin) overnight at 4℃. The membranes were washed with 1×TBST and incubated with diluted HRP-conjugated secondary antibody for 1 h at room temperature with shaking. After being washed with 1×TBST, the membranes were developed with ECL western blotting detection reagent, and analyzed with ImageJ system (Clinx, China).

2.12 Statistical Analysis

One-way ANOVA was used for statistical analysis followed by the Tukey's *post hoc* test using GraphPad Prism 8.0 (GraphPad Software, USA). The results were shown as means±standard error of mean (SEM). A value of $P<0.05$ was considered to be statistically significant. All the experiments were carried out independently in triplicate.

3 Results

3.1 Cytotoxicity of ECPs on Hepa1-6 Cells

The viability of cells exposed to ECPs was determined by MTT method, with the results illustrated in Figs.1A and B. When ECPs≥0.16 mg mL^{-1} for 24 h and ≥0.04 mg mL^{-1} for 48 h, cell viability decreased with increased ECPs concentration. According to the cell viability, the IC_{50} values of ECPs towards Hepa1-6 cells were found to be 0.2048 mg mL^{-1} for 24 h and 0.1219 mg mL^{-1} for 48 h. LDH leakage assay was also employed to evaluate the cytotoxicity of ECPs. As shown in Figs.1A and B, significant cytotoxic effects were caused by ECPs starting from a concentration of 0.2 mg mL^{-1} for 24 h, and 0.04 mg mL^{-1} for 48 h.

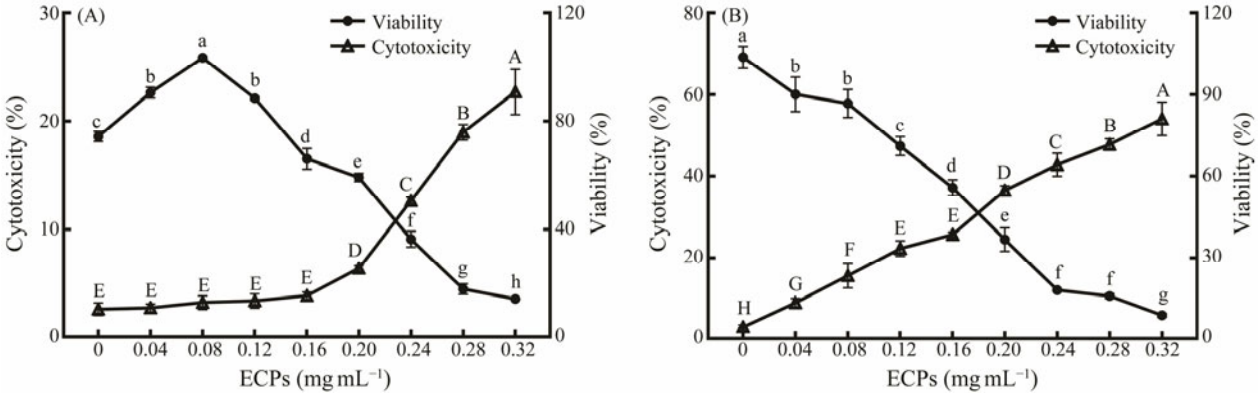

Fig.1 Cytotoxic effects of ECPs on Hepa1-6 cells. Cells were exposed to ECPs (0–0.32 mg mL^{-1}) for 24 h (A) and 48 h (B). Cell viability was determined using the MTT assay. Cytotoxic was detected by LDH assay. Each value represents mean± SEM of three independent experiments. Different letters indicate significant differences between different samples ($P<0.05$).

3.2 Effect of ECPs on Apoptosis in Hepa1-6 Cells

To identify whether ECPs induce apoptosis, Hepa1-6 cells were stained with Annexin V-FITC/PI reagents and examined for morphological changes under fluorescence microscope. As shown in Fig.2A, typical morphological changes (lower density of flat round wrinkled adherent cells, more floating cells, nuclear pyknotic rupture, formation of apoptotic bodies) were observed in cells after exposure to ECPs for 24 h in a dose-dependent manner. The quantification of cell apoptosis was also performed using flow cytometry. As illustrated in Fig.2B, ECPs treatment significantly increased the population of apoptotic cells. In the non-exposed cells, 3.85% were positive for Annexin V-FITC staining, while ECPs exposure dose-dependently caused 18%–48% apoptotic cells. These results further confirmed the ability of ECPs to induce apoptosis in Hepa1-6 cells.

3.3 Effect of ECPs on ROS Generation in Hepa1-6 Cells

Cell apoptosis can be induced by ROS (Li *et al*., 2018), and polyphenols have been shown to generate oxidative stress in cancer cells (Kim *et al*., 2001; Sundarraj *et al*., 2020). Thus, we examined the effect of ECPs on ROS production in Hepa1-6 cells by DCFH-DA staining, with the aim to explore the mechanism of ECPs-induced cell death. As observed in Figs.3A and 3C, ECPs treatment significantly enhanced intracellular ROS by 17%–47% in a dose-dependent manner, as indicated by greater green fluorescence in cells post exposure than that of control.

3.4 Effect of ECPs on Mitochondrial Membrane Potential (MMP) in Hepa1-6 Cells

The destruction of mitochondrial integrity is one of the landmark events in the early stage of apoptosis (Chen *et al*., 2017). Mitochondrial membrane potential (Δψm) and mitochondrial morphology were assessed to elucidate the effects of ECPs on mitochondrial function. As shown in Figs. 3B and 3D, ECPs treatment remarkably increased in green fluorescence and decreased in red fluorescence in a dose-dependent manner. To be more specific, cells exposed to ECPs for 24 h showed notably decreased fluorescence ra-

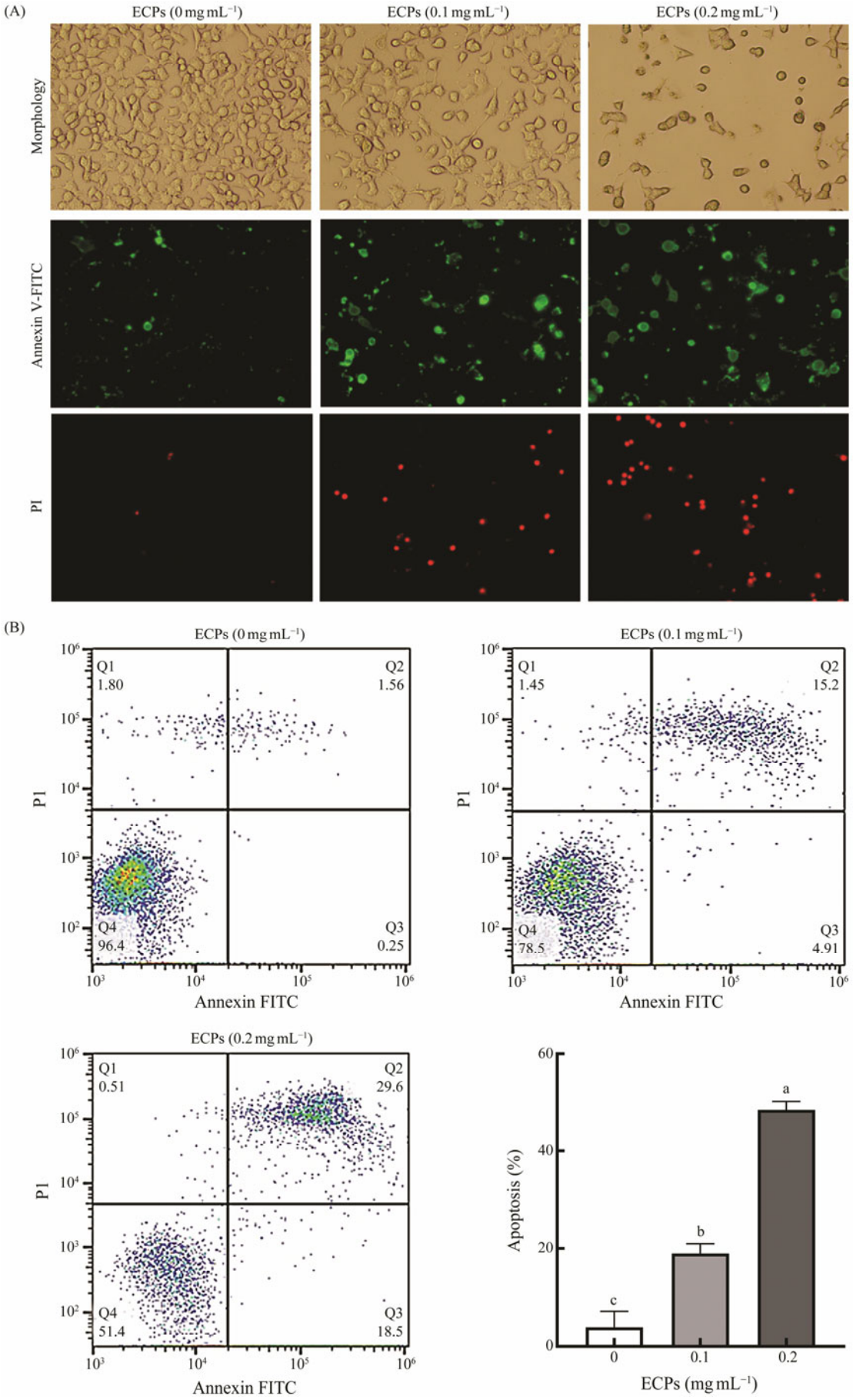

Fig.2 Effect of ECPs on apoptosis in Hepa1-6 cells. Hepa1-6 cells were treated for 24 h with ECPs (0.1 and 0.2 mg mL^{-1}). Cell apoptosis was evaluated by fluorescence microscopy at 200× (A), and flow cytometry (B) using Annexin V-FITC/PI double staining assay. Different letters indicate significant differences between different samples ($P<0.05$).

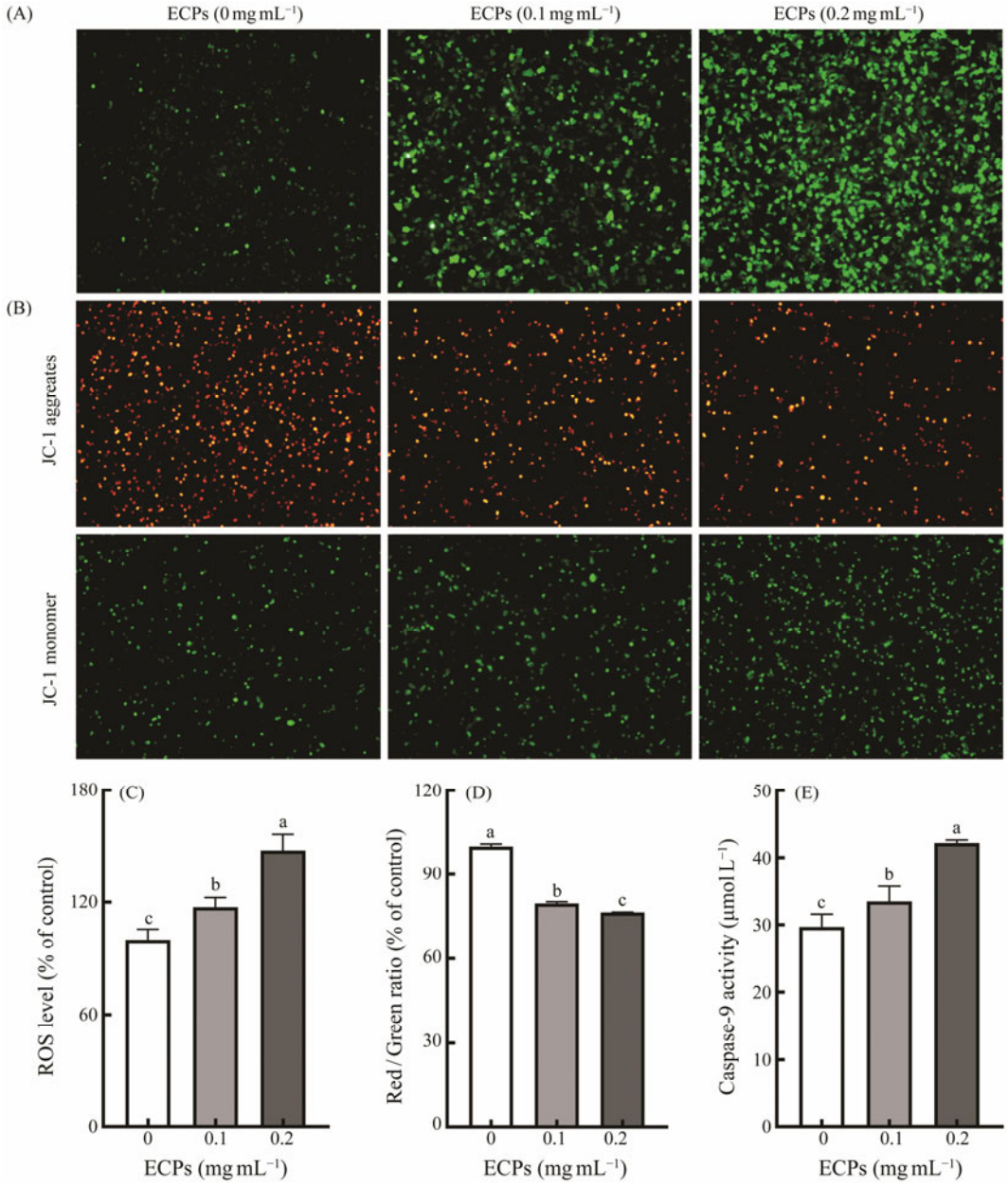

Fig.3 Effects of ECPs on ROS level (A and C), MMP (B and D) and Caspases-9 (E) in Hepa1-6 cells. Cells were exposed to ECPs (0.1 and 0.2 mg mL^{-1}) for 24 h. Intracellular ROS levels were monitored by measuring the fluorescence intensity of DCFH-DA using fluorescence microscopy (A) and a microplate reader (C). The level of MMP was evaluated by determining the fluorescence intensity of JC-1 using fluorescence microscopy (B) and a fluorospectrophotometer (D). Caspase-9 activation in Hepa1-6 cells was evaluated using microplate reader (E). Each value represents mean ± SEM of three independent experiments. Different letters indicate significant differences between different samples ($P<0.05$).

tios (red/green), which were 79.65% at 0.1 mg mL^{-1} and 76.40% at 0.2 mg mL^{-1}, in comparison with control group, indicating the capacity of ECPs in destroying MMP in Hepa1-6 cells.

3.5 Effect of ECPs Caspase-9 Activation in Hepa1-6 Cells

Caspase-9 is an important upstream caspase during apoptosis signal transduction, triggering apoptosis cascade reactions (Zhang *et al*., 2021). We therefore examined the effect of ECPs on caspase-9 activation in Hepa1-6 cells. As shown in Fig.3E, ECPs induced an 1.15-1.42 fold increase of caspase-9 activity.

3.6 Effects of ECPs on Protein and Gene Expression of Apoptosis-Related Molecules

To further elucidate the mechanism of ECPs-induced apoptosis in cancer cells, the protein levels of apoptosis-associated molecules (cytochrome c, Bcl-2, Bax and cleaved caspase-3) were analyzed by western blot. As in Fig.4, in comparison with non-exposed cells, those treated with ECPs had significantly lower Bcl-2 protein expression (0.1 mg mL^{-1}-0.76, 0.2 mg mL^{-1}-0.41) while notably higher protein levels of Bax (0.1 mg mL^{-1}-1.56, 0.2 mg mL^{-1}-2.30), cytochrome c (0.1 mg mL^{-1}-1.46, 0.2 mg mL^{-1}-1.98) and cleaved-caspase 3 (0.1 mg mL^{-1}-1.39, 0.2 mg mL^{-1}-2.37).

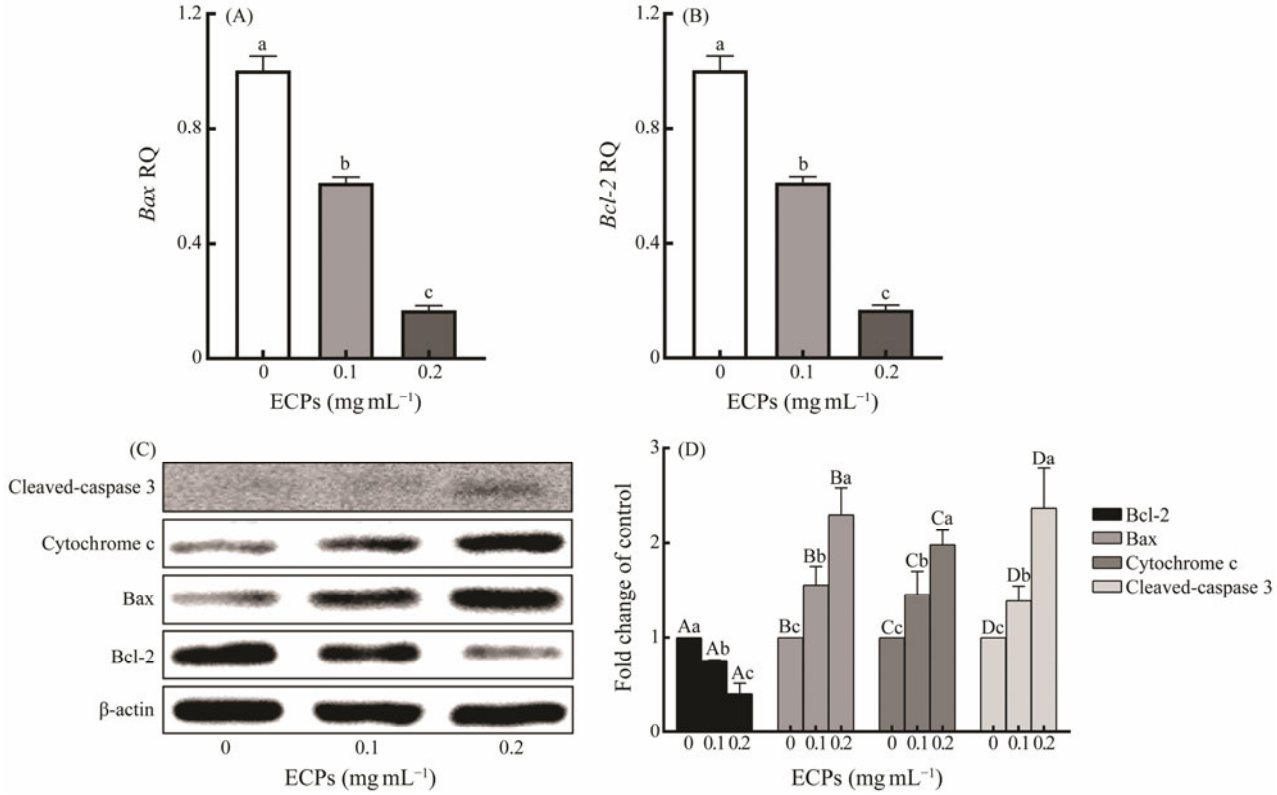

Fig.4 Effects of ECPs on gene expression and protein levels in Hepa1-6 cells. Gene expression of *Bax* (A) and *Bcl-2* (B) was analyzed by qPCR using the ddCT method. The expression of Bax, Bcl-2, cytochrome c and caspase-3 proteins were detected by Western blot (C and D). Each value represents mean ± SEM of three independent experiments. Different letters indicate significant differences between different samples ($P<0.05$).

The relative gene expression of *Bcl-2* and *Bax* were also quantified. Compared to the control (1.00), the mRNA level of *Bcl-2* gene was significantly down-regulated in ECPs-treated cells (0.1 mg mL^{-1}-0.61, 0.2 mg mL^{-1}-0.17). By the contrast, *Bax* gene expression was significantly higher in the exposure group (0.1 mg mL^{-1}-1.53, 0.2 mg mL^{-1}-2.75) than that in the control group (1.01).

4 Discussion

Induction of cancer cell apoptosis (the programmed cell death) has been recognized as an important method in cancer therapy. Phenolic compounds derived from marine alga have shown great potential as alternative anti-cancer reagents (Yuan *et al*., 2005; Nwosu *et al*., 2011; Karadeniz *et al*., 2015; Kosanić *et al*., 2015). In this study, we evaluated the anti-cancer property of ECPs and the underlying mechanisms using murine Hepa1-6 hepatoma cells. It was found that ECPs inhibited cell growth in a concentration-dependent manner, with an IC_{50} value of 0.2048 mg mL^{-1} for 24 h. Cytotoxic effects on cancer cells have also been reported in natural products derived from other algae, including the acetone extracts of green algae *Ulva lactuca* and *Enteromorpha intestinalis* on LS174, A549, Fem-x and K562 cell lines (Kosanić *et al*., 2015), extract from *Ulva rigida* on A375 melanoma cell line (Barreto *et al*., 2012), and ethanol extract of brown alga *Laminaria japonica* on BEL-7402 cell line (Karadeniz *et al*., 2015), extract from *Fucus spiralis* on A375 melanoma cell line (Barreto *et al*., 2012). To determine whether the cytotoxicity of ECPs was due to apoptosis, ECPs-treated cells were examined under the fluorescence microscope, and typical morphological changes were observed including cytoplasmic shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, and apoptotic body formation. Flow cytometry results demonstrated that ECPs exposure significantly elevated the proportion of apoptotic population, further confirming that ECPs induced apoptosis in Hepa1-6 cells.

At present, there are three known apoptosis pathways, including intrinsic (mitochondrial) pathway, extrinsic (death receptor) pathway and endoplasmic network pathway, and the intrinsic pathway is the universal apoptosis mechanism (Wang *et al*., 2011). The intrinsic pathway is triggered within the cell by a variety of stimuli such as environmental changes, drugs, genetic damage, hypoxia, and oxidative stress (Li *et al*., 2012). In response to the stimuli, mitochondria open the permeability transition pore, releasing cytochrome c, a pro-apoptotic protein, into the cytoplasm (Lee *et al*., 2008). Released cytochrome c activates caspase-9, and the activated caspase-9 cleaves and activates caspase-3 (Thornberry *et al*., 1998; Hengartner *et al*., 2000). As apoptosis executioner, the activated caspase-3 initiates apoptotic DNA fragmentation by cleaving the inhibitor caspase-activated DNAase (Dvorakova *et al*., 2002; Hess *et al*., 2007). ROS is tightly linked to activation of intrinsic (mitochondrial) pathway (Xu *et al*., 2014; Liu *et al*., 2016). Reactive oxygen species (ROS) can target and modify the protein components of the mitochondrial permeability tran-

sition pore (MPTP) complex, leading to MPTP opening and subsequent collapse of mitochondrial transmembrane potential (Δψm) (Agudo-López *et al*., 2011; Redza-Dutordoir *et al*., 2016). The findings of this study showed increased ROS generation, Δψm collapse, elevated release of mitochondrial cytochrome c, enhanced caspase-9 activity and up-regulated caspase-3 expression in ECPs-treated Hepa1-6 cells, strongly suggesting apoptosis driven by mitochondria, which was induced by ECPs. Apoptosis induced by the mitochondria has also been found in polyphenols from other algae and land plants. For instance, *dieckol*, isolated from brown alga *Ecklonia stolonifera* induced apoptosis in Hep3B cells *via* the activation of both death receptor and mitochondria-dependent pathways (Yoon *et al*., 2013). A variety of terrestrial polyphenols, such as *foxtail millet bran* (Shi *et al*., 2015), *Fisetin* (Sundarraj *et al*., 2020), *Butein* (Kim *et al*., 2001), *artichoke* polyphenols (Mileo *et al*., 2012) caused apoptosis of HCT-116 cells, HepG2 cells, human leukaemic HL-60 cells and human breast cancer MDA-MB 231 cell line through activation of mitochondria-mediated intrinsic pathways, respectively.

Very often, disruption of mitochondrial transmembrane potential accompanies altered expression of proteins belonging to Bcl-2 family. These proteins are either proapoptosis (*e.g*., Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) by promoting release of cytochrome c, or anti-apoptosis (*e.g*., Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) by blocking such release (Liu *et al*., 2012; Navazani *et al*., 2021). It should be noted that it is the balance between the pro- and anti-apoptosis molecules, rather than the absolute quantity, that determines the initiation of apoptosis (Burguillos *et al*., 2011; Mahdavi *et al*., 2018). As shown in our results, the transcription and translation of Bcl-2 members (*i.e*., Bcl-2, Bax) changed in cells post ECPs exposure. ECPs treatment dose-dependently decreased Bcl-2 expression at both gene and protein levels, whereas significant elevation in Bax levels was observed. The Bcl-2 family also plays a central role in other terrestrial polyphenol-induced mitochondrial apoptosis pathways. For instance, exposure to artichoke polyphenols (Mileo *et al*., 2012), curcumin (Chiu *et al*., 2009) and carvacrol (Arunasree *et al*., 2010) caused apoptosis on MDA-MB231 cells *via* a molecular mechanism correlated to an increase in the *Bax*/*Bcl2* protein ratio. The anti-cancer activity of tea polyphenols (epigallocatechin-3-gallate, EGCG) was also shown to be associated with the modified expression of Bcl-2 family proteins (*i.e*., up-regulated Bax and down-regulated Bcl-2) and tumor suppressor gene p53, as observed in different cancer cells (bladder, breast, pancreatic, esophageal and prostate) (Hastak *et al*., 2005; Qin *et al*., 2007; Shankar *et al*., 2007; Liu *et al*., 2017; Moradzadeh *et al*., 2017).

To sum up, our results suggest that ECPs could be a novel natural therapeutic reagent against liver cancer, *via* induction of mitochondrial apoptosis. Currently, the work on characterization and evaluation of individual bioactive constituent(s) from ECPs are underway in our laboratory. In addition, *in vivo* tumor suppressing activity of ECPs is to be investigated to provide accurate and systemic analysis of their anti-cancer function.

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