Current Research in Food Science 5 (2022) 904–917

Contents lists available at ScienceDirect

Current Research in Food Science

journal homepage: www.sciencedirect.com/journal/current-research-in-food-science

Research Paper

Proanthocyanidin oligomers extract from hawthorn mediates cell cycle arrest, apoptosis, and lysosome vacuolation on HCT116 cells

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ARTICLE INFO

Handling editor: Alejandro G. Marangoni

Keywords:
Hawthorn proanthocyanidin oligomers extract
Colon cancer
Cell cycle
Apoptosis
Vacuolation
Lysosomes

ABSTRACT

In this study, Hawthorn oligomic procyanidins extracts (HPOE) were evaluated for their anticancer activity on colorectal cancer. Our results showed that HPOE arrested HCT116 cells cycle at G2/M phase through P53-Cyclin B pathway and promoted apoptosis partly via mitochondrial (Caspase 9-Caspase 3) and death receptor (Caspase 8-Caspase 3) pathways. Meanwhile, it was found that HPOE aggravated HCT116 cells death through lysosomal vacuolation, which was verified by inhibitor/activator of P53-ILC3 signaling pathway. Taken together, HPOE exerted anticancer effects which lays the foundation for the development of functional foods for clinical colon cancer patients.

1. Introduction

Colorectal cancer is one of the top three malignancies in the world with high mortality and morbidity. This gastrointestinal cancer often occurs at the junction of rectum and sigmoid colon, mainly caused by abnormal proliferation of intestinal mucosal epithelial cells, and is related to obesity, lack of physical exercise, alcoholism, and another unhealthy lifestyle (Potter, 1999). With the improvement of living standards, colorectal cancer incidence is increasing year by year, which is related with the stealthiness of early colorectal cancer symptoms making the detection difficult, thus results into failure of timely surgical treatment (Akimoto et al., 2021). According to the World Health Organization (WHO), about 19300000 people (including 865630 Females and 1065960 Males) were diagnosed with colorectal cancer and over 9000000 people (including 419536 Females and 515637 Males) have died all over the world until now (https://gco.iarc.fr/). These phenomena implied that further advances in the treatment of colorectal cancer and establishment of new therapeutic platforms are necessary.

The anticancer drugs are generally studied from two perspectives: inhibiting proliferation and promoting cell death (Nirmala and Lopus, 2020; Chen et al., 2020). As for cell proliferation, Cyclins (Cyclin A, B, C, D and E) and the subsequent cyclin-dependent kinases (Cdks) are reported for their critical roles in the progression of cell division cycle (Malumbres and Barbacid, 2009); As for cell death, many accumulating evidences demonstrate that cell death could be classified into apoptotic cell death (intrinsic and extrinsic pathway), necrotic cell death (membrane rupture and inflammation related pathway), and other forms of cell death (Autophagy, Iron, Starvation, et al. related pathway) (Chen et al., 2018). Generally, these studies provide theoretical basis for anti-colorectal cancer drugs development.

Nowadays, with the emergence of toxicity, drug resistance and other problems of clinical chemotherapy drugs, a variety of new anticancer drugs have emerged (Lin et al., 2016). Natural products have become raw materials for anti-cancer drugs development due to their abundant resources (Liu et al., 2021), clear anti-tumor activities, low toxicity to normal tissues and resistance to drugs (Yuan et al., 2017). Hawthorn is a member of rosaceae family cultivating in East Asia, North American and Europe consisting about 280 species (Kim et al., 2022; Li et al., 2022). To date, more than 150 chemical compounds have been defined from the leaves, flowers and berries of Hawthorn including flavonoids, lignans, monoterpenoids, triterpenoids, and organic acids (Teng et al., 2020; Wu

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https://doi.org/10.1016/j.crfs.2022.05.009
Received 20 April 2022; Received in revised form 12 May 2022; Accepted 20 May 2022
Available online 29 May 2022

Abbreviation

HPOE	Hawthorn Proanthocyanidin Oligomers Extract
WHO	World Health Organization
Cdks	Cyclin-dependent kinases
Pif	Pifthrin-α
FMK	Z-VAD-FMK
DMSO	Dimethyl sulfoxide
Baf	V-ATPase inhibitor Bafilmycin A1
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
LDH	Lactate Dehydrogenase
TEM	Transmission Electron Microscopy
LC-ESI-MS	High Perforkjvmance Liquid Chromatography-Electrospray Tandem Mass Spectrometry

et al., 2014). Some studies have presented that Hawthorn extracts could ameliorated hepatic injury, lipid accumulation, inflammation, and so on. Among them, Hawthorn Proanthocyanidin Oligomers Extract (HPOE) were less studied, and most of them are known for their antioxidant activity (Kim et al., 2022). It has been proved that proanthocyanidin could alleviate intestinal function through interacting with gut microbiota (Wu et al., 2021). However, the effects of HPOE directly on colon have not been explored yet.

Based on these assumptions, the present study was planned to investigate the anti-tumor effect of HPOE on HCT116 cells. And, we reported that HPOE arrested HCT116 cell cycle at G2/M, promoted apoptosis progress partly through Caspase 9/8-Caspase 3, and activated lysosomal vacuolation mediated cell death through P53-ILC3. These findings would provide insights into the development of HPOE related products with regulating intestinal function.

2. Materials and methods

2.1. HPOE extraction

The method of HPOE extraction was carried out according to the method reported in our previous study (Teng et al., 2020; Yin et al., 2011).

As for HPOE extraction, firstly, hawthorn flesh, originated from Xinglong County of Hebei Province in China, was soaked with ethanol (Solarbio, China) to obtain ethanol extract of hawthorn proanthocyanidin. Then, the oligomers of hawthorn proanthocyanidin were separated by ethyl acetate (Solarbio, China) extraction method. Finally, AB-8 macroporous resin (Solarbio, China) was used for further separation and purification, and hawthorn proanthocyanidin oligomers with high purity were obtained (Yin et al., 2011; Xie et al., 2022).

2.2. LC-ESI-MS analysis

The main components of HPOE were chromatographed by LC-ESI-MS on a 3.0 mm × 100 mm × 2.7 μm EC-C18 column with Agilent 12 60 Series LC/MSD Trp System (Agilent Technologies, Palo Alto, USA) at a flow rate of 0.5 mL/min at 280 nm. The mass spectrometry conditions were based on ESI mode, under the condition of ion source gas temperature of 300 °C, gas flow rate of 0.2 mL/min, atomizer pressure of 50 psi, capillary voltage of 3500 V. Negative ion mode, and the scanning range of 100–2000 m/z (Zhou et al., 2014; Fang et al., 2022).

2.3. Cell culture and drug treatment

HCT116 cells (Shanghai Wei Atlas Biological Technology Co., Ltd.) were co-cultured with or without (250, 300, 350 μg/mL) HPOE for 48 h period. Further, p-53 inhibitor Pifithrin-α (Pif, Beyotime, China) (Lin et al., 2016), Caspases inhibitor Z-VAD-FMK (FMK, Beyotime, China) (Lin et al., 2016), V-ATPase inhibitor Bafilmycin A1 (Baf, MedChemExpress, China) (Hino et al., 2020) were added 0.5 h prior to HPOE treatment. Both the cell types (suspension and adherent cells) were harvested finally for other experiments.

2.4. Cell viability

For this, HCT116 cells were seeded into a 96-well plate at a density of 8×10^4 cells/well and incubated at 37 °C, 5% CO_2, and 95% humidity until 80% confluence. Cells were treated with gradient concentration of HPOE (0, 75, 150, 225, 300, 375, 450, 525, and 600 μg/mL) dissolved by DMSO. After 24 h/48 h cultivation, 3-(4, 5-dimethylthiazol-2-yl) −2, 5-diphenyltetrazolium bromide (MTT) assay was performed (Sun et al., 2020). For lactate dehydrogenase (LDH) cytotoxicity assay, manufacturer's instructions were followed as mentioned on the LDH Cytotoxicity Assay Kit (Beyotime, China) (Zhang et al., 2022).

2.5. Hoechst 33342/PI staining

This staining is based on the fact that chromatin gets shrunk and permeability of cells membrane increases when cells undergo apoptosis. Hoechst33342 can penetrate apoptotic cells membrane and stain the nucleus, while PI cannot cross intact cell and cells with incomplete membrane can only be stained (Crowley et al., 2016). After HPOE treatment of HCT116 cells, cells were collected in 1.5 mL tubes and re-suspended in a staining buffer (Solarbio, China). Following the instructions, 5 μL Hoechst 33342 and 5 μL PI were added and thoroughly mixed. The excess dye was washed away with PBS after centrifugation. Antifade mounting medium (Beyotime, China) was added for visualization using inverted fluorescence microscope (model number: CKX31, Olympus, Japan) (Guo et al., 2022).

2.6. Annexin V-FITC/PI double-stained assay

Annexin V-FITC bound apoptotic HCT116 cells were stimulated by 488 nm wavelength and detected at 525 nm. PI stained damaged HCT116 cells nuclei were also exited at 488 nm wavelength, and probed at 650 nm. Annexin V-FITC positive cells presented the early or late apoptosis stage apoptosis in HCT116 cells (Sun et al., 2020; Zhai et al., 2018; Wu et al., 2021).

2.7. Cell cycle analysis

According to the protocol mentioned in Cell Cycle and Apoptosis Analysis Kit (Beyotme, China), cell cycle analysis was performed. In brief, trypsin digested HCT116 cells were terminated in cell culture medium. After centrifugation at 1000*g* for 3–5 min, the supernatant was removed. 70% ethanol was used to fix HCT116 cells at 4 °C for 30 min. The fixed HCT116 cells were stained with PI solution at 37 °C for 30 min. Data capturing and analysis were performed using flow cytometry (Beckman Coulter, USA) (Liu et al., 2012; Chen et al., 2021).

2.8. Protein extraction and western blot

These specific operation steps were performed following the previous study with some modification (Sun et al., 2020). The specific

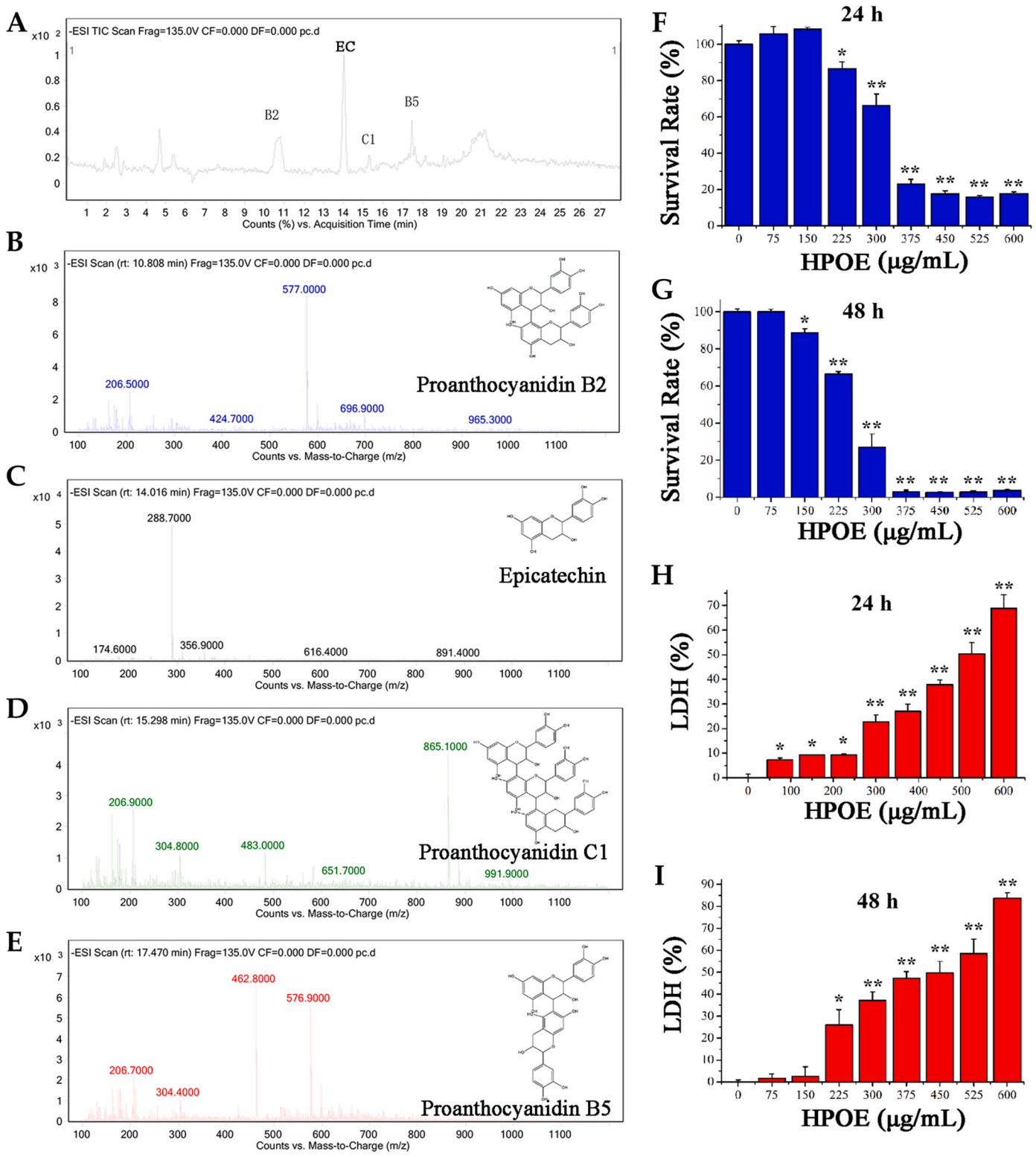

Fig. 1. HPOE inhibits cell proliferation of HCT116 cells.
The LC-ESI-MS of hawthorn proanthocyanidin oligomers. A, Total spectrometry; B, Proanthocyanidin B2; C, Epicatechin; D, Proanthocyanidin C1; E, Proanthocyanidin B5. Cell proliferation inhibition as assayed by MTT at 24 h (F) and 48 h (G). Lactate dehydrogenase release rate at 24 h (H) and 48 h (I). The IC_{50} value occurred in the concentration ranges between 250 and 350 μg/mL of HPOE. Bars in (F), (G), (H), and (I) represent SE, $^{*}p < 0.05$ or $^{**}p < 0.01$ versus non-treated control of HCT116 cells.

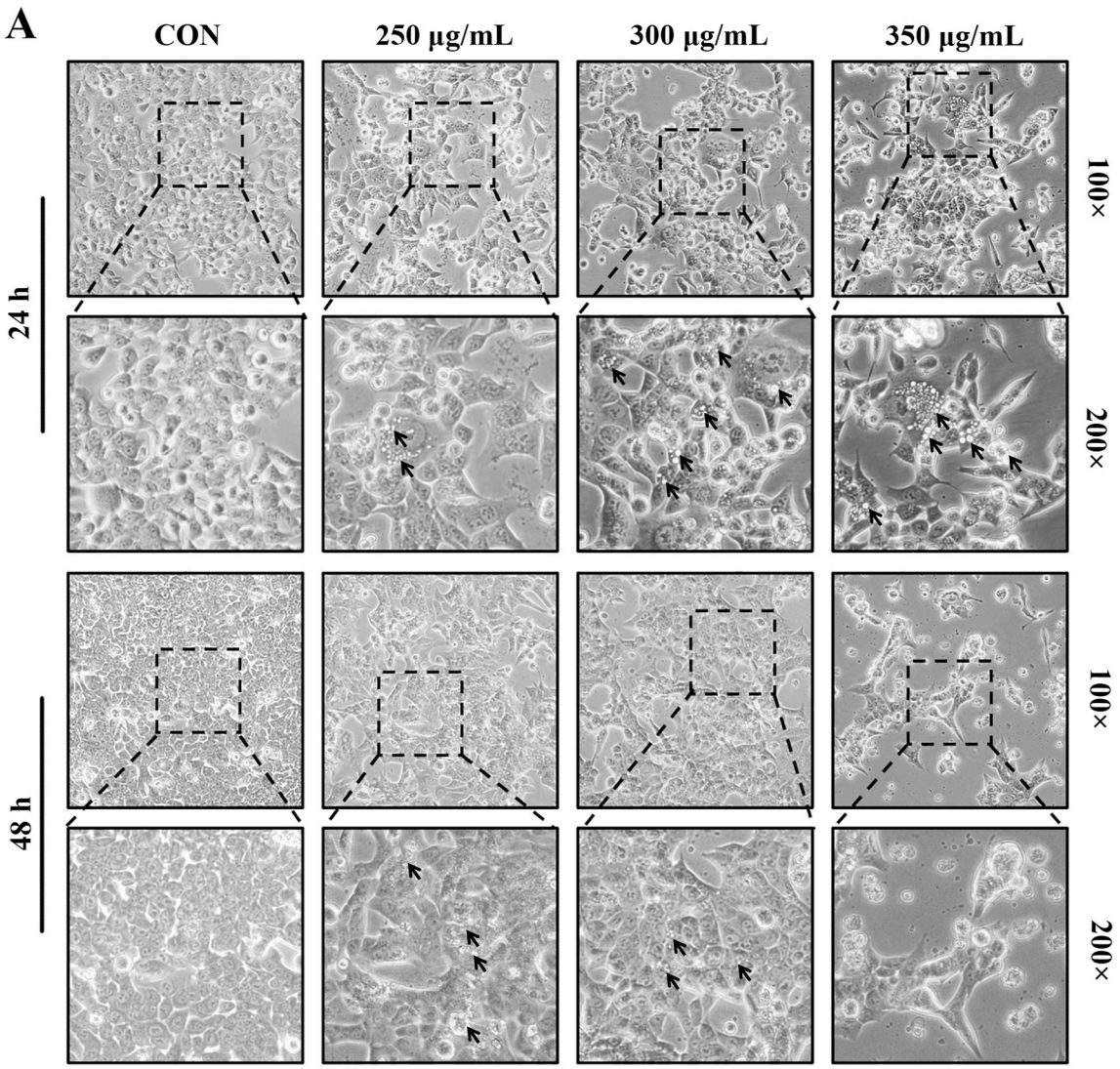

Fig. 2. Cellular morphology of HCT116 cells after HPOE treatment.
The morphological changes of HCT116 cells upon 24 h or 48 h treatment with 250, 300, and 350 μg/mL HPOE. The cells images depict 100 × and 200 × magnifications using an electron microscope.

binding sites were incubated with anti-Cycline D antibody (1:5000, Proteintech, USA), anti-Cyclin A antibody (1:500, Proteintech, USA), anti-Cyclin B antibody (1:1000, Proteintech, USA), anti-P53 antibody (1:500, Proteintech, USA), anti-Caspase 9 antibody (1:200, Proteintech, USA), anti-Caspase 8 antibody (1:100, Proteintech, USA), anti-Caspase 3 antibody (1:200, Proteintech, USA), anti-ILC3 antibody (1:1000, Proteintech, USA), anti-β-actin antibody (1:5000, Proteintech, USA), respectively. After incubation with anti-mouse/rabbit lgG HRP-linked secondary antibody (1:5000, Proteintech, USA), image and analysis were detected by ImageQuant LAS 4000 mini (GE Healthcare, China).

2.9. Transmission electron microscopy (TEM)

HCT116 cells (with or without HPOE for 0, 6, 12, 24, and 48 h treatment) were fixed with 2.5% glutaraldehyde at 4 °C overnight and post-fixed with 1% OsO_4 for 2 h. After removing the OsO_4 waste solution, samples were washed with 0.1 M, pH 7.0 PBS 15 min. Then, the samples were dehydrated with gradient concentration ethanol solution (30%, 50%, 70%, 80%, 90%, and 95%), embedded by epoxy resin, and heated at 70 °C overnight. Finally, sample slices were cut by LEICA EM UC7 (LEICA, U.S.A) about 70 nm, stained with uranyl acetate and lead citrate before observation by JEM 1200EX transmission electron microscope (JEOL, Japan). Nikon digital camera (Dmx1200F, Nikon, Japan) was used to capture and analyze the images (Hino et al., 2020).

2.10. Endoplasmic reticulum, mitochondria, lysosome fluorescence staining

After HPOE treatment, hanks' balanced salt solution with Ca^{2+} & Mg^{2+} was used to wash the treated HCT116 cells followed by fixation

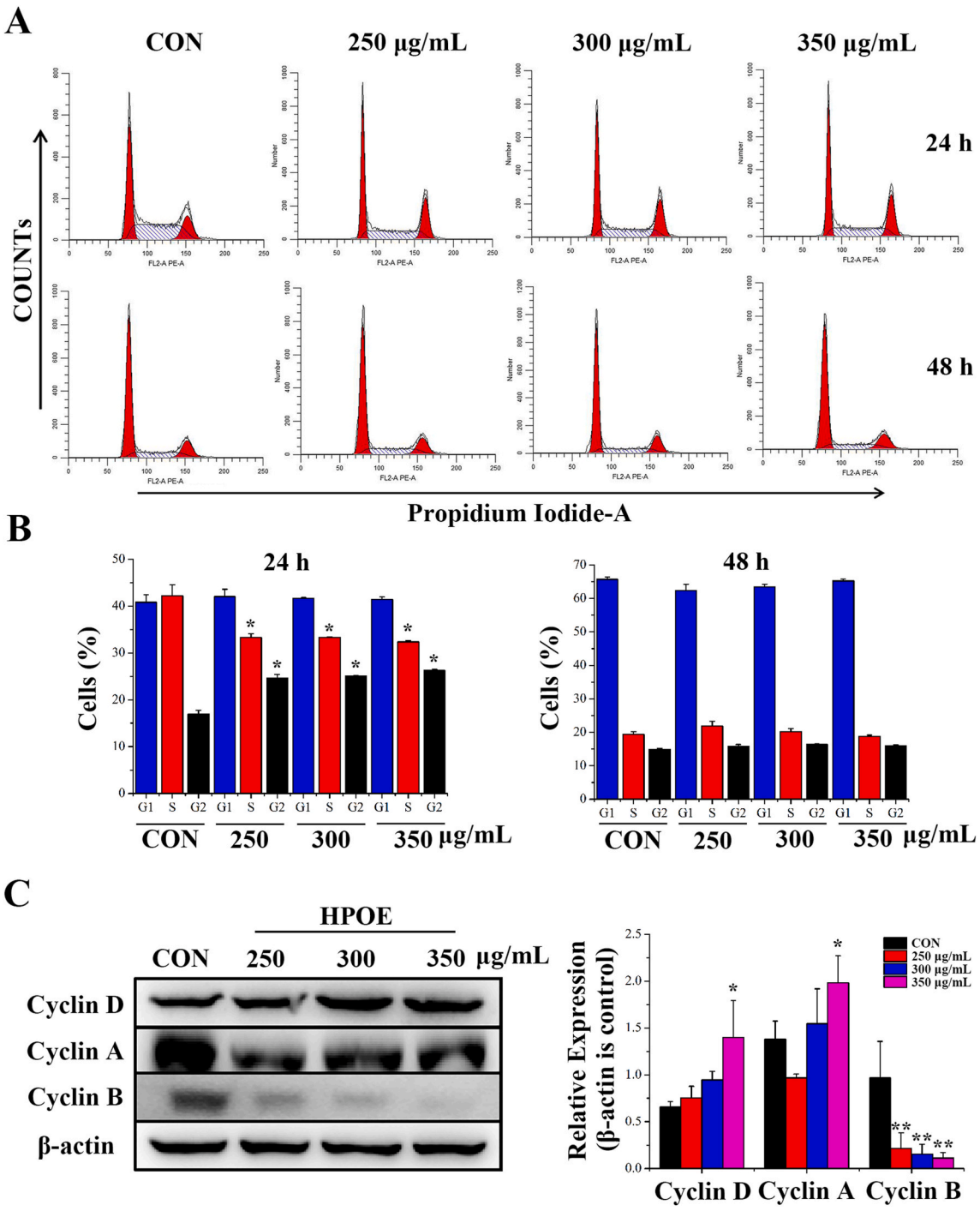

Fig. 3. HPOE inhibits cell cycle progression at G2 phase in HCT116 cells.
(A) HCT116 cells were treated with 250, 300, and 350 μg/mL of HPOE for 24 h or 48 h and the percentage of cells in distinct phases of cell cycle was analyzed; (B) The histogram represented the quantitative result of triple independent experiments in (A); (C) The total protein was subjected to immunoblotting of Cyclin D, Cyclin A and Cyclin B. Cyclin D and Cyclin A were upregulated, wherease Cyclin B was downregulated in HCT116 cells in a dose-dependent manner. Bars in (B) and (C) represent SE, $*p < 0.05$ or $**p < 0.01$ versus non-treated control of HCT116 cells.

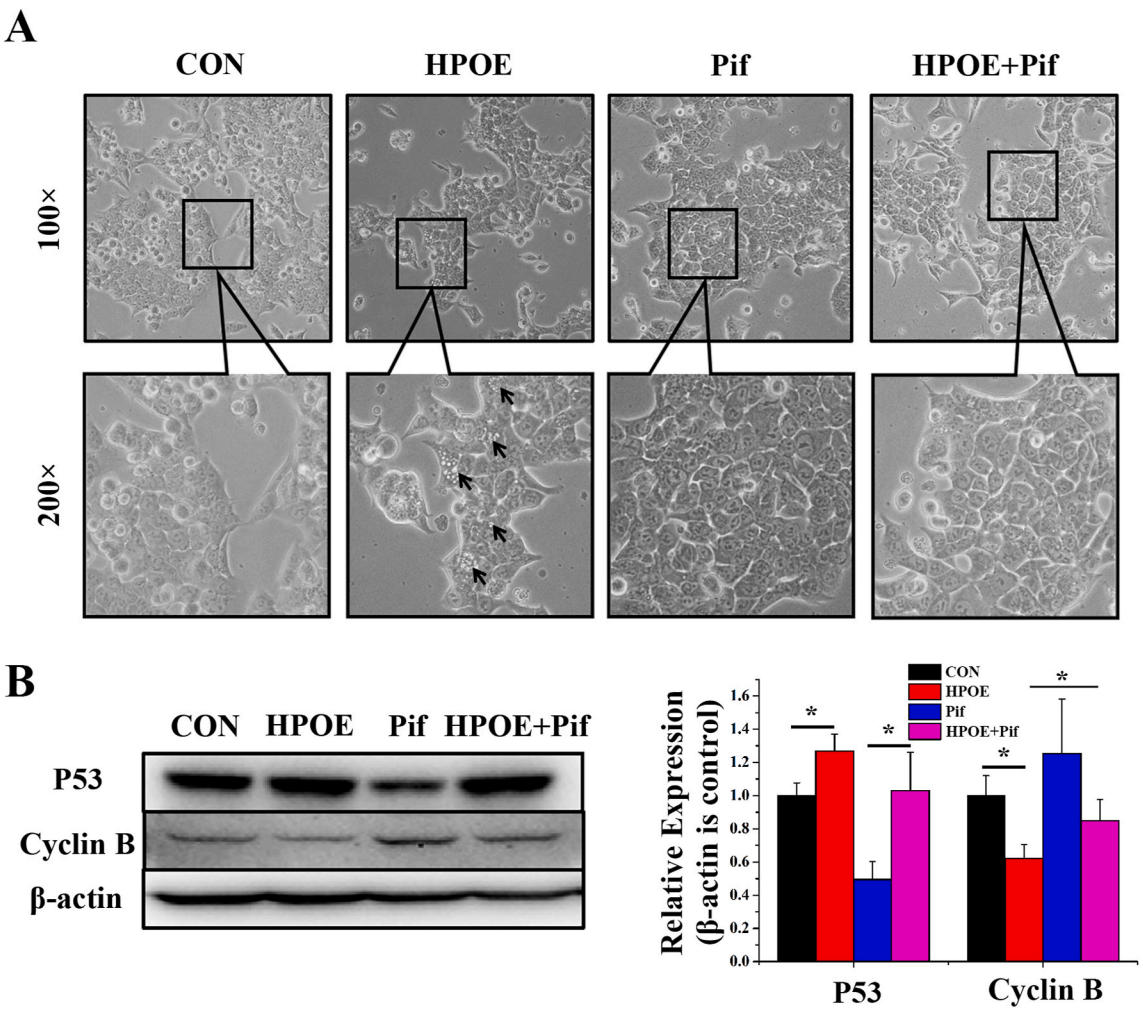

Fig. 4. HPOE arrests cell cycle of HCT116 cells at G2 phase through P53-Cyclin B pathway.
Pif (P53 inhibitor) was treated half an hour before HPOE treatment. (A) The morphological changes of CON, HPOE, Pif, and Pif + HPOE. (B) The protein expression of P53 and Cyclin B in HCT116 cells after culturing with HPOE and P53 inhibitor Pif. Bars in (B) represent SE, $*p < 0.05$ or $**p < 0.01$ versus non-treated control of HCT116 cells.

using 4% formaldehyde for 2 min and staining with Mito-Tracker Red (Beyotime, China), ER-Tracker Red (Beyotime, China) or Lyso-Tracker Red (Beyotime, China) and observation using fluorescence microscope (Olympus, Japan) (Gao et al., 2021).

2.11. Experimental data analysis

All the data in this study are presented as the means ± standard error (SE). The Origin 8.6 statistical software was used for data analysis, and the significance levels of each group were determined by one-way ANOVA and finally denoted by $*p < 0.05$, $**p < 0.01$.

3. Results

3.1. The inhibitory effect of HPOE on HCT116 cells

After extraction, separation, and purification of hawthorn, highly purified hawthorn proanthocyanidin oligomers were obtained. To identify the main components of complex proanthocyanidin mixtures, High Performance Liquid Chromatography- Electrospray Tandem Mass Spectrometry (LC-ESI-MS) was used for qualitative and quantitative analysis (Fig. 1A). The results showed that the crude proanthocyanidin extracts contained 21.80% Proanthocyanidin B_2 (Fig. 1B), 36.43% Epicatechin (Fig. 1C), 10.95% Proanthocyanidin C_1 (Fig. 1D), and 7.49% Proanthocyanidin B_5 (Fig. 1 E). Some reports have showed that procyanidins could be absorbed at small intestine and interact with colonic microflora to produce low molecular weight phenolic acids (Phenyl Valerolactone, Phenylacetic Acids et al.) (Zhang et al., 2016). Most of procyanidins (58–78%) could be excreted from urine, but have significant inhibitory effects on gastrointestinal carcinogenesis (Zhang et al., 2016; Bitzer et al., 2015). To detect the effect of HPOE on colon cancer, MTT assay (Fig. 1F and G) and LDH cytotoxicity assay (Fig. 1H and I) were performed on HCT116 cells. As shown in Fig. 1F and G, the 50% inhibition rate of HPOE on HCT116 cells ranged from 250 μg/mL to 350 μg/mL after 24 h or 48 h treatment. As for the cellular morphology (Fig. 2 A), HPOE decreased the cell number and intensified the degree of cell shrinkage in a time- and dose-dependent manner. These results indicated that proanthocyanidin oligomers from hawthorn could inhibit colon cancer HCT116 cells proliferation.

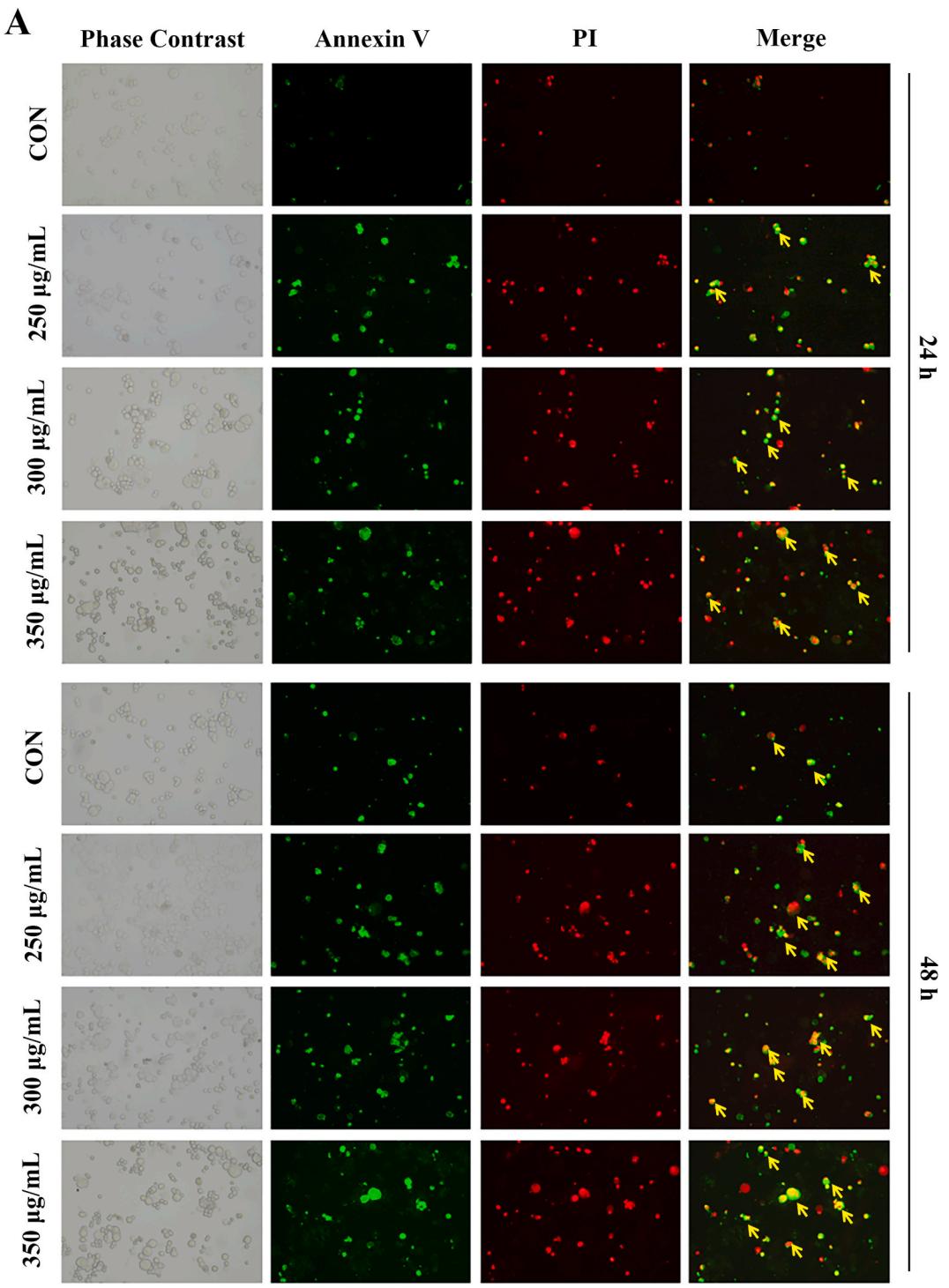

Fig. 5. The Hoechst 33342/PI staining of HCT116 cells after HPOE treatment.
To validate the apoptosis of HCT116 cells after treatment with various concentrations of HPOE (250, 300, and 350 μg/mL) for 24 h or 48 h at 100 × magnification.

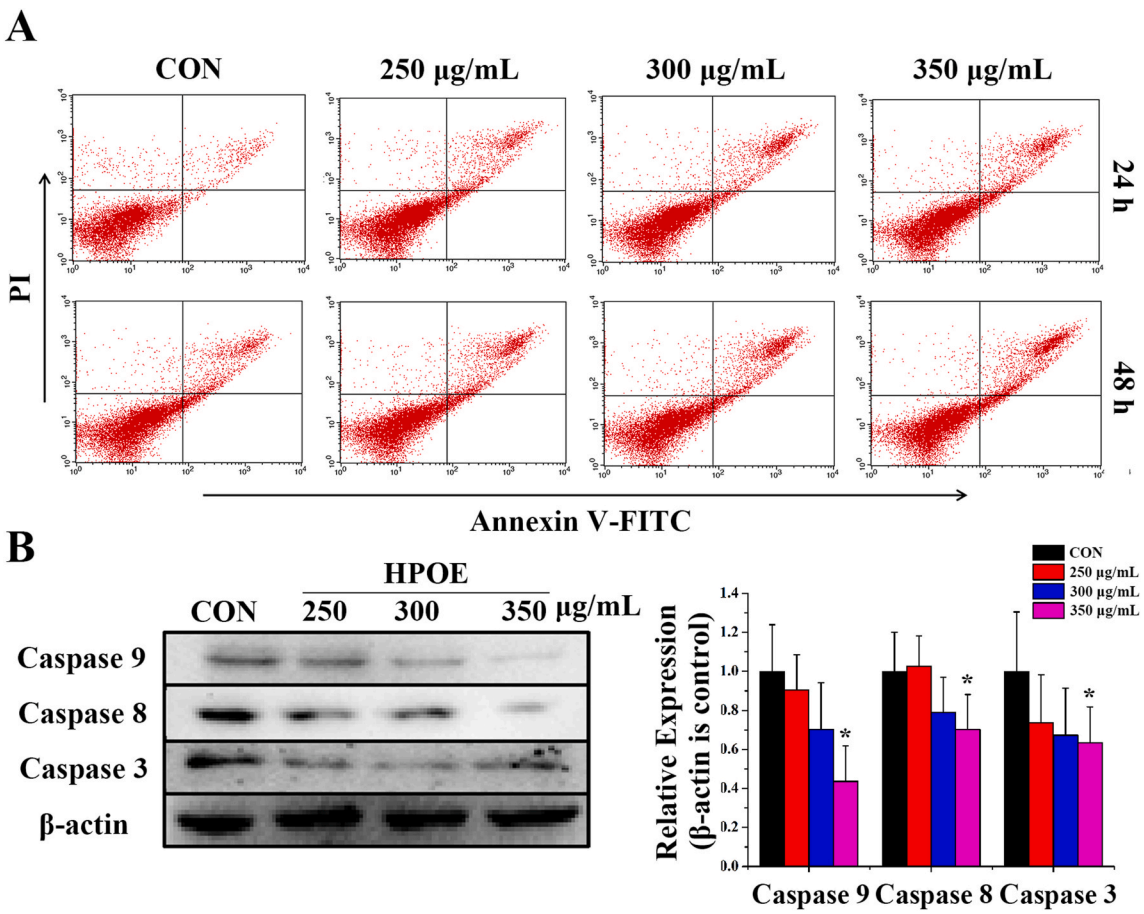

Fig. 6. HPOE promotes HCT116 cells apoptosis in a dose-dependent manner.
(A) After double-staining with Annexin-V FITC and PI, flow cytometry was used for the analysis of HCT116 cells following with different concentrations of HPOE addition. (B) The protein expression levels of Caspase 9, Caspase 8, and Caspase 3 were detected by Western blot analysis, (β-actin as a control). Data in (B) were presented as mean ± SE, $*p < 0.05$ or $**p < 0.01$.

3.2. HPOE arrested cell cycle at G2 phase through P53-Cyclin B pathway

To reveal the possible underlying mechanisms involved in growth inhibition, the cell cycle of treated HCT116 cells was examined. HCT16 cells were treated with 250, 300, and 350 μg/mL of HPOE for 24 h and 48 h respectively, and the changes of cell distribution in cell cycle arrest were detected by flow cytometry (Fig. 3 A). The quantitative results showed that HPOE increased the percentage of cells at G2/M phase in a dose-dependent manner after 24 h treatment, but not after 48 h treatment (Fig. 3 B). As for the cell cycle is regulated by the synthesis and destruction of Cyclins, Cyclin D (G1 Phase), Cyclin A (S Phase) and Cyclin B (G2/M Phase) were further detected from protein levels by Western Blot in 24 h group (And and Walker, 1999). Fig. 3 C showed that HPOE inhibited the expression of Cyclin B from 77% to 88%, while HPOE increased Cyclin D (about 113% comparing with CON group) and Cyclin A (about 43% comparing with CON group) expression. These results indicated that HPOE mainly arrested HCT116 cells at G2/M phase. To establish the molecular mechanisms of HPOE-induced G2/M cell cycle arrest in HCT116 cells, we screened out P53 which could inhibit Cyclin B transcriptional expression (Krause et al., 2000). The expression of P53 was silenced by Pifithrin-α (Pif, P53 inhibitor), but the P53 and Cyclin B could still be significantly affected, which implied that HPOE arrested HCT116 cells at G2/M phase partly through P53-Cyclin B pathway (Fig. 4A and B).

3.3. HPOE promoted HCT116 cells apoptosis via caspase 9 and caspase 8

Apoptosis is another possible potential mechanism of HPOE on HCT116 cells growth inhibition. Fig. 5 presented the overlapped area of red fluorescent part and green fluorescent part with the increasing HPOE dosage and processing time extension, which indicated the increasing degree of shrunk chromatin and cell membrane permeability. The present results in Fig. 6 A indicated that HPOE promoted late apoptotic cell death at 24 h. Comparing with the number of apoptotic cells detected at 24 h, HPOE increased the apoptotic cell number dramatically after 48 h treatment. These phenotype results indicated that HPOE could promote HCT116 cells apoptosis. To explore the molecular mechanisms of HPOE on apoptotic protein expression, Western blot was performed to

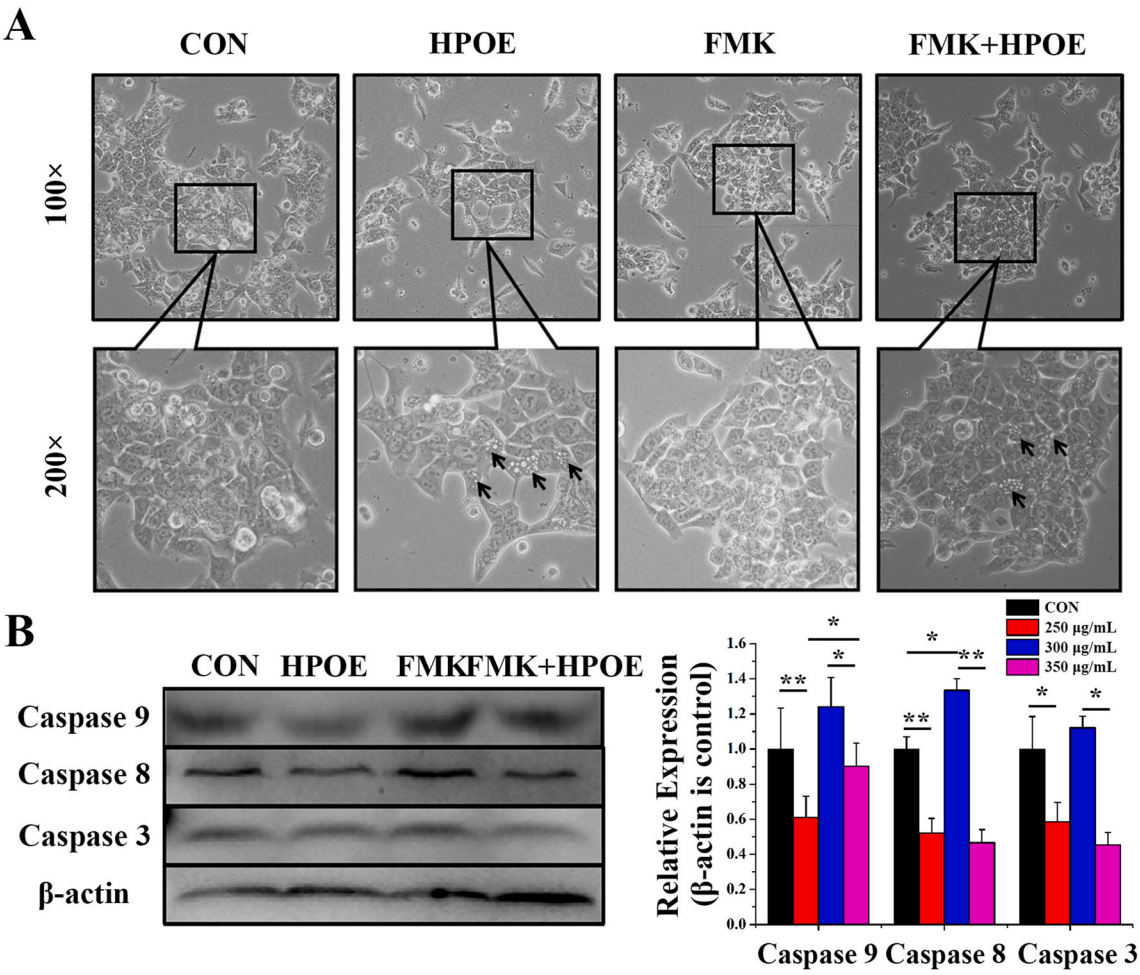

Fig. 7. HPOE enhances HCT116 cells apoptosis partly through Caspase 9/8-Caspase 3 pathway.
FMK (Caspases inhibitor) was treated half an hour before HPOE treatment. (A) The morphological changes of HCT116 cells were captured by electron microscope. (B) The protein expression levels of Caspase 9, Caspase 8, and Caspase 3 were detected by Western blot assay, (β-actin as a control). Data in (B) were presented as mean ± SE, $*p < 0.05$ or $**p < 0.01$.

evaluated the translation levels of Caspase 9 in mitochondria pathway (Allan and Clarke, 2009), Caspase 8 death receptor signaling pathway (Tummers and Green, 2017), and Caspase 3 the common downstream regulator of these two signaling pathways (Wu et al., 2011). HPOE down-regulated Caspase 9, Caspase 8, and Caspase 3 in a dose-dependent manner (Fig. 6 B), and the Caspases inhibitor Z-VAD-FMK could not observably reverse its effect (Fig. 7A and B). Collectively, this result indicated that HPOE could enhance HCT116 cells apoptosis partly through Caspase 9/8-Caspase 3 pathway.

3.4. HPOE inhibited lysosomal mediated death partly through P53-ILC3

As for cell morphology observation, we were surprised to find out that multiple vacuoles were formed in cytoplasm after HPOE treatment for 24 h and disappeared after 48 h treatment (Fig. 2). To explicit the detailed formation process of the cytoplasmic vacuoles, TEM was performed to record the vacuoles morphology at 0, 6, 12, 24, and 48 h. HPOE promoted the vacuoles enlargement until covering the entire cytoplasm and eventually leading to HCT116 cells death (Fig. 8 A). Not only that, partial magnification of HCT116 cells showed that some cytoplasmic vesicles contain many little pieces inside (Fig. 8 B). Some studies have proven that the vacuolation could be derived from all membranous organelles, such as endoplasmic reticulum, mitochondria, and lysosomes (Shubin et al., 2016). The fluorescent probe staining of endoplasmic reticulum (Fig. 9 A), mitochondria (Fig. 9 B) and lysosome (Fig. 9 C) showed that only some lysosomes could be stained by lyso-tracker red (Fig. 9 C). These results suggested that HPOE-induced vacuoles in HCT116 cells originated from lysosomes.

It has been reported that the inhibitor (Bafilomycin A1, Baf) of vacuolar-type ATPase could completely inhibit vacuole formation (Hino et al., 2020). We pretreated HCT116 cells with Baf, and found out that HPOE could not induce vacuoles formation (Fig. 10 A). In this study, we also observed that culturing HCT116 cells with Bif (P53 inhibitor) before HPOE treatment could also inhibit the vacuoles development (Fig. 4 A). Considering these results, we pretreated HCT116 cells with Baf and Bif, and tested the protein level of P53 and ILC3. After Baf treatment, HPOE

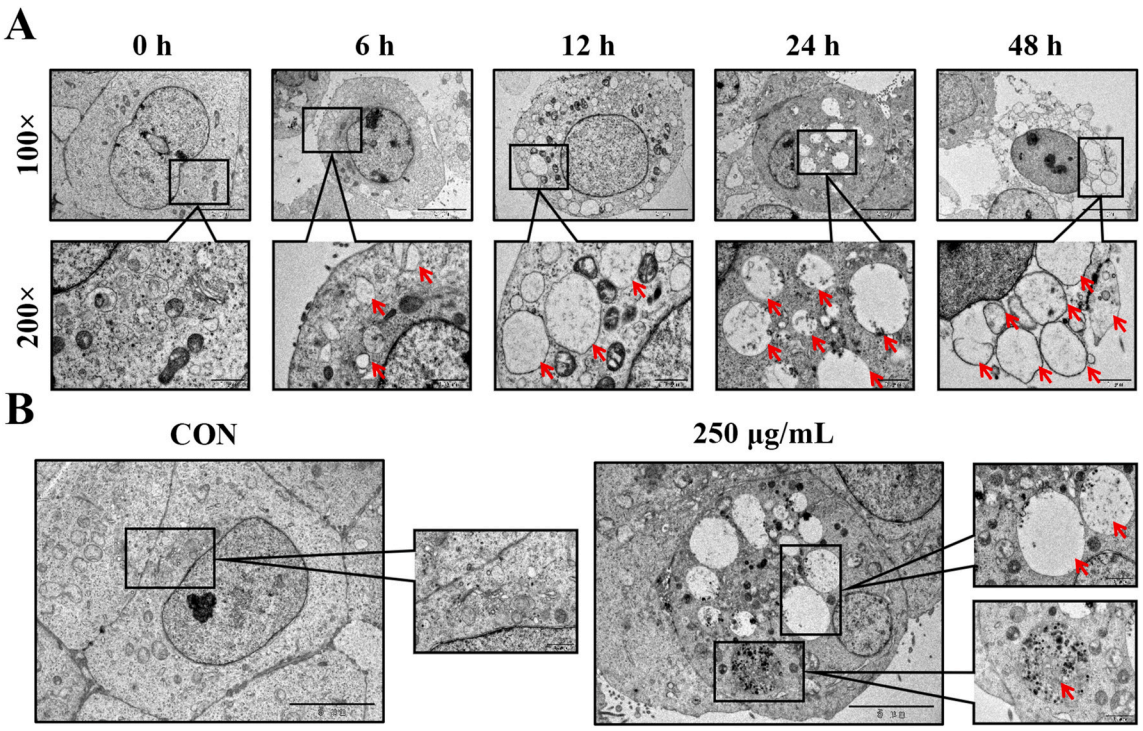

Fig. 8. The transmission electron microscopy images of untreated or HPOE treated HCT116 cells.
(A) The vacuolization of HCT116 cells with the change of time (0, 6, 12, 24, and 48 h) after HPOE treatment. (B) The contents in vacuoles versus non-treated control of HCT116 cells.

could reverse ILC3 expression to a certain extent, but not for P53 (Fig. 10 B). However, the expression of repressed P53 and ILC3 was relatively less reversed after Bif treatment (Fig. 10 B). To summarize, these results suggested that HPOE induced vacuoles derived from lysosomes aggravating HCT116 cells death via P53-ILC3 pathway.

4. Discussion

Accompanied with the increasing epidemiological and experimental evidences, diets rich in flavonoids, most of which are originated from plants, are likely to be associated with the risk reduction of colorectal cancer (Grosso et al., 2017; Kim et al., 2020). Proanthocyanidin, consisting with flavan-3-ol subunits, have low oral bioavailability. However, on the one hand, proanthocyanidin could be partly absorbed at stomach and small intestine, and on the other hand, interact with colonic microflora and colon cells, and ultimately play a protective role on intestinal tract (Zhang et al., 2016; Serra et al., 2010). Our previous studies already explored the absorption rate of HPOE at small intestine and its effect on diversity of gut microbes (data not published), but the relationship between hawthorn proanthocyanidin and colon cells has not been established. Hence, the inhibitory mechanisms of HPOE on colon cancer was studied using HCT116 cells.

From previous studies, the dose of HPOE treating HCT116 cells was not clear. Few studies have been reported on the Hawthorn Proanthocyanidin Oligomers extracted from hawthorn leaves or flowers, and the concentrations of these extract culturing cells ranged from 1.1 μg/mL to 250 μg/mL (Krajka-Kuźniak et al., 2014; Chai et al., 2014; Zumdick et al., 2012; Li et al., 2018). The reason for such a wide range of concentration may be related to the extraction materials, extraction methods, cell culture methods, and so on. In this study, we extracted HPOE from the fresh flesh. Although HCT116 cells were still cultured with the crude extract of hawthorn proanthocyanidin, we estimated the amount of each oligomic proanthocyanidins by LC-ESI-MS (Fig. 1 A), which would provide reference for the later experiment's repeatability and the study of synergistic effects of olligomic proanthocyanidins.

To reveal the mechanisms of HPOE on colon cancer, we first detected the cell cycle arrest of HCT116 cells by cell cytometry and found that HPOE induced G2 phase cells accumulation (Fig. 3 A) through P53-Cyclin B pathway (Fig. 4 B). Then, effects of HPOE on apoptosis were investigated from two aspects of classic mitochondrial- and death receptor-related signaling pathways (Tummers and Green, 2017; Carneiro and El-Deiry, 2020). As shown in Fig. 7, HPOE could promote apoptosis through Caspase 9/8, but not solely dependant on these two signaling pathways. During the process of exploring mechanisms, we were surprised to find that the number and volume of these vacuoles increase in HPOE dose-dependent manner within 24 h, but this phenomenon disappeared after 48 h treatment, and the number of dead cells rapidly increased (Fig. 2). Therefore, we hypothesized that the formation of vacuoles may be another mechanism by HPOE induced HCT116 cells death. Some studies have statemented the cell death modes associated with vacuolation include paraptosis, methuosis, necroptosis, and oncosis (Zheng et al., 2016; Ritter et al., 2021; Dhuriya and Sharma, 2018; Zhang et al., 1998), and these vacuoles may be derived from all the membranous organelles in the cell, such as mitochondria, endoplasmic reticulum, lysosome, etc (Shubin et al., 2016). The immunofluorescence staining results showed that lysosomes and vacuoles were co-located (Fig. 9), which led us to search for lysosomal related death regulation mechanisms. Bafilomycin A1 is vacuolar-type ATPase (a proton pump across the lysosomal membrane) inhibitor which was associated with the development of lysosomal origin vacuoles (Hino et al., 2020). Considering that Pifithrin-α could also inhibit vacuoles formation (Fig. 4 A), Western blot was used to discuss the upstream and downstream relationship between P53 and ILC3, and it was finally confirmed that HPOE could inhibit lysosomal mediated death partly through P53-ILC3 (Fig. 10B and C).

To conclude, our study revealed that HPOE could inhibit HCT116 cells proliferation through P53-Cyclin B, partly activating both mitochondrial- and death receptor-related pathways, and promote lysosomal swelling related cell death through P53-ILC3. Although we could present the anti-colon cancer mechanisms of HPOE, future studies are warranted

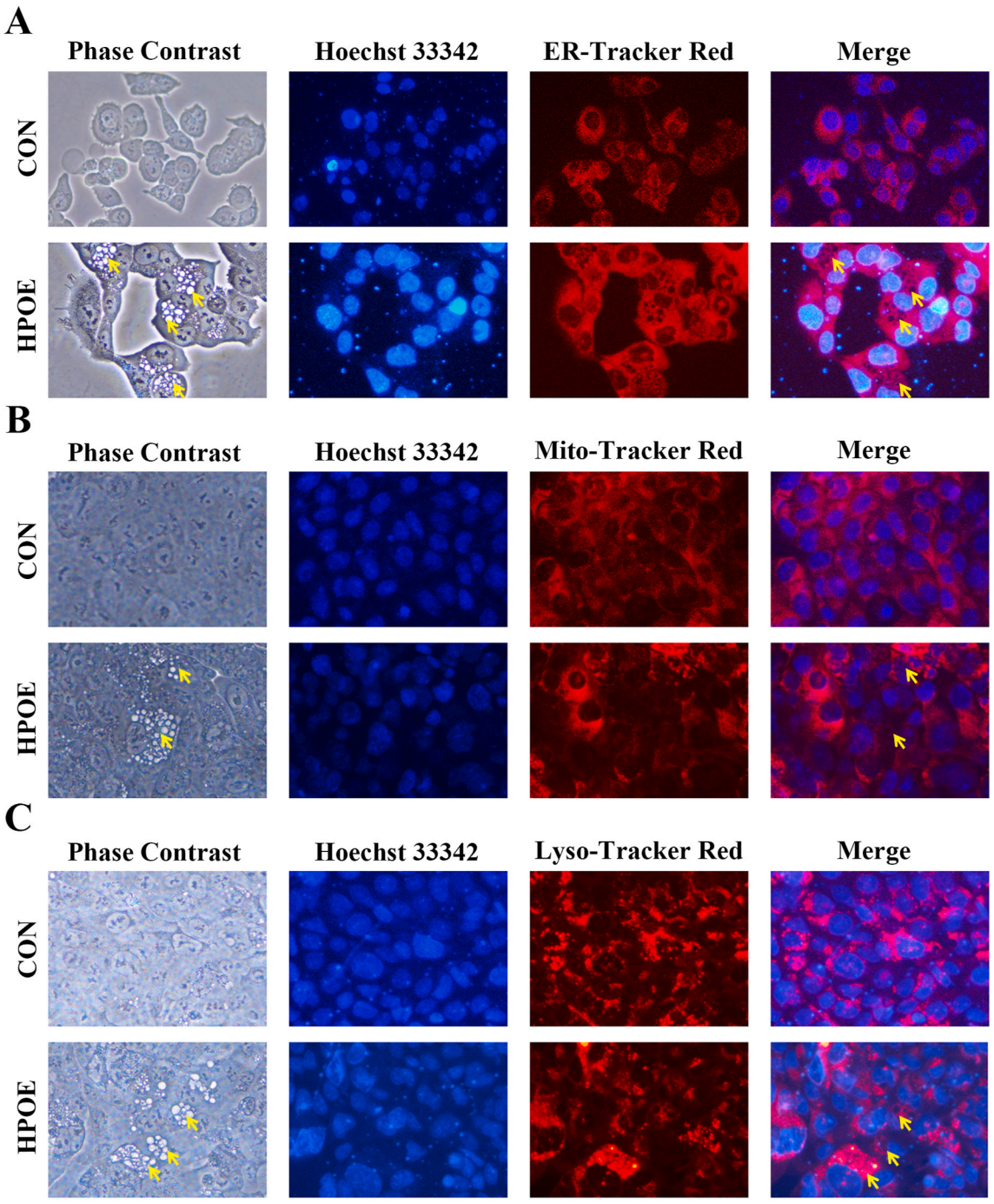

Fig. 9. HPOE promotes the vacuoles development partly from lysosome.
The fluorescent probe staining of endoplasmic reticulum, mitochondria, lysosome in HCT116 cells with or without HPOE (300 μg/mL) supplement.

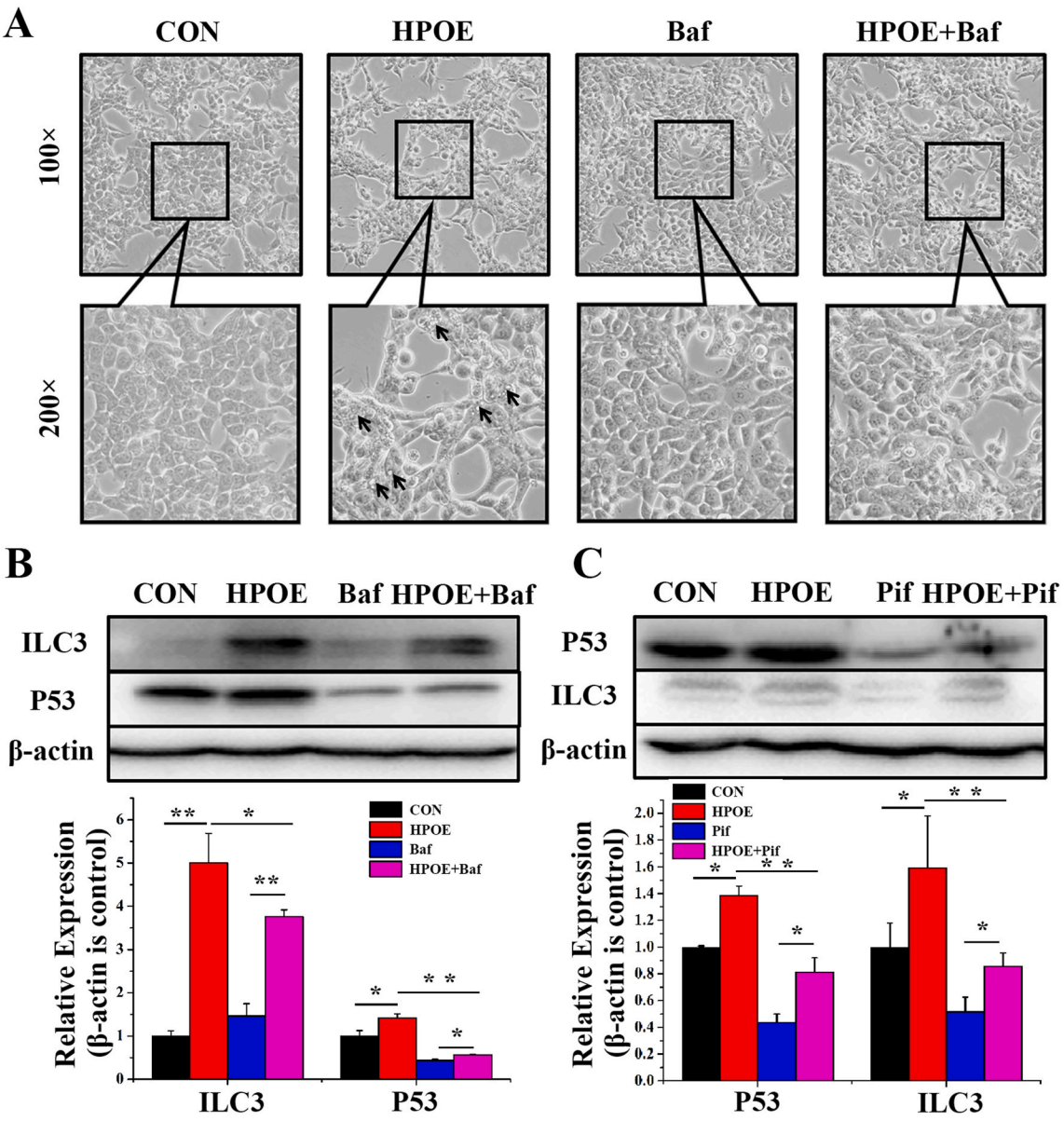

Fig. 10. HPOE inhibits cytoplasmic vacuole formation through P53-ILC3 pathway.
The morphological changes of HCT116 cells after treating with or without Baf (vacuolar-type ATPase inhibitor), (B) the protein levels of ILC3 and P53 were evaluated by Western blot, β-actin as a control. (C) The relationship between P53 and ILC3 from translation level, β-actin as a control. Bars in (B) and (C) represent SE, $*p < 0.05$ or $**p < 0.01$.

to clearly state the anticancer effects of hawthorn proanthocyanidin mixture as well as synergistic effects of each hawthorn proanthocyanidin.

Funding

This research was supported by the Introduced Talent Research Project of Hebei Agricultural University (YJ2020029), the Natural Science Foundation of Hebei Province (No.C2021204152), the Youth talent cultivation project of North Minzu University (2021KYQD27, FWNX14), and the Key research and development projects in Ningxia Province (2021BEF02013).

CRediT authorship contribution statement

Ya-Sai Sun: Investigation, Writing – original draft, All the authors listed above have read and approved to the version of the final manuscript. **Zi-Wei Wang:** Investigation, Methodology, All the authors listed above have read and approved to the version of the final manuscript. **Zhe Gao:** Formal analysis, Conceptualization, All the authors listed above have read and approved to the version of the final manuscript. **Wen Zhao:** Supervision, Resources, All the authors listed above have read and approved to the version of the final manuscript. **Kiran Thakur:** Writing – original draft, Writing – review & editing, All the authors listed above have read and approved to the version of the final manuscript. **Qian Zhong:** Resources, Software, All the authors listed above have read and approved to the version of the final manuscript. **Zhao-Jun Wei:** Resources, Writing – original draft, Writing – review & editing, All the

authors listed above have read and approved to the version of the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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