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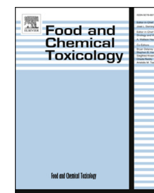

Pomegranate peel extract polyphenols induced apoptosis in human hepatoma cells by mitochondrial pathway

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ABSTRACT

This study was aimed to investigate the influence of pomegranate peel polyphenols (PPPs) on the proliferation and apoptosis of HepG2 cells (a kind of human hepatoma cells) and the related mechanism. The inverted fluorescence microscope and the flow cytometer (FCM) were used to test the changes of the cellular morphology, cell cycle, apoptosis, reactive oxygen species (ROS) and mitochondrial transmembrane potential ($\Delta\psi$m). The kit was used to measure the activities of caspase-3/9, and Western Blot was used to detect the expressions of apoptosis-associated proteins including p53, Bcl-2/Bax, Cyt-c and PARP. The results showed that the cells cycle of HepG2 arrested at the S-phase by PPPs and the amount of the early apoptotic cells and ROS level were increased obviously, the level of Cyt-c and the activity of Caspase-3/9 markedly were also increased by PPPs, as well as the ratio of Bax/Bcl-2 and the protein expressions of P53. It was concluded that PPPs could inhibit the growth of HepG2 cells by blocking the cell cycle and inducing the mitochondrial apoptotic pathway in a dose-dependent manner.

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1. Introduction

Hepatic carcinoma is the sixth most common human cancer in the world and the third most commonly diagnosed malignant tumor in China (Li et al., 2013). Primary liver cancer is one of the most common malignant tumors clinically (Ji et al., 2008), owing to it can transfer in the early stage inside the liver, once hepatic carcinoma is discovered, and it would be in the middle or late stage. In the past 10 years, the global incidence of liver cancer has not been effectively controlled (Bartek et al., 2004; Van Delft and Huang, 2006). The present study showed that the growth and proliferation of tumor cells were closely related to the level of apoptosis (Larrosa et al., 2006). Meanwhile, looking for components with the effects of inducing apoptosis of tumor cells in natural products is particularly important for the treatment of liver cancer (Yang et al., 2012).

Pomegranate with a long history of cultivation is a kind of plants which is available for medicine and food in China (Zhao et al., 2014). Pomegranate polyphenols, which can be used for antioxidant, antitumor, regulating blood fat and other healthy and medicinal treatment, exist widely in peel, pulp and seeds of pomegranate (Shaban et al., 2013). Especially mentioned that the content of polyphenols is enough high in the Pomegranate peel. Currently, pomegranate juice, pomegranate wine and other relative products in the market are popular. However, during the process of producing, the 20%–30% of pomegranate peel containing much more polyphenols is discarded causing the waste of the large raw materials (A. Ferrueloa et al., 2014). Pomegranate polyphenols are very beneficial to health and are known for the possession of the remarkable antioxidant properties capable on protecting normal cells from various stimuli-induced oxidative stress and cell death. However, the studies on the anticancer effects of PPPs and its main active components as well as its mechanism were rarely reported. Therefore, this study systematically and intensively researched the anticancer activity and mechanism of the PPPs with a positive significance in a thorough study of the edible and medicinal care to provide theoretical basis so as to fully exploit the pomegranate.

2. Materials and methods

2.1. Materials and reagents

Human hepatoma cell HepG2: purchased from the Experimental Animal Center of Fourth Military Medical University cell bank. Incubator (Thermo,USA). The Roswell Park Memorial Institute-1640 (RPMI-1640) medium was purchased from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was

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purchased from Zhejiang Tianhang Biological Technology Co. (Zhejiang, China). Dimethyl sulfoxide (DMSO), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Hochest33258 staining, propidium iodide (PI), RNase-A were purchased from Sigma (St. Louis, MO, USA). Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) kit and DCFH-DA were purchased from Nanjing Jiancheng biology Co. (Nanjing, China). Caspase-3 and Caspase-9 detection kit were purchased from Beyotime Biotechnology Co. (Shanghai, China). All the other cell culture reagents were purchased from Sinopharm (Beijing, China).

2.2. Pomegranate peel polyphenols (PPPs) extraction

2.2.1. Preparation of PPPs

Pomegranate fruits were purchased from local markets in Lintong, Shaanxi province of China. The peels were manually removed, sun-dried and finely powdered. For the extraction of polyphenol compounds, ultrasonic extraction was used. Briefly, 1.0 g dried powder was weighed and then put into a 250 mL conical flask. The optimal conditions of extraction polyphenol compounds were as follows: the concentration of ethanol was 60% (v/v), the ultrasonic extraction time was 25 min and ultrasonic power was 100 W, the ratio of material to liquid was 1:20. The extraction solution was centrifuged at 4000 g for 20 min. The resulting supernatant was collected and then concentrated by rotary evaporator at 40 °C, followed by vacuum freeze-drying to get crude extracts. The dried resin (25 mg) and crude extracting solution (1 mg/mL) of pomegranate peel polyphenols were blended in a conical flask and then oscillated for 10 h. After adsorption, the supernatant was discarded and the remaining fraction was eluted with distilled water twice. After that, the same volume 70% (v/v) ethanol was put into the conical flask and the mixture was oscillated for 10 h to desorb. Furthermore, the resulting solution was concentrated and followed by vacuum freeze-drying to obtain refined extracts of pomegranate peel polyphenols which were maintained at −20 °C in darkness until analyzed.

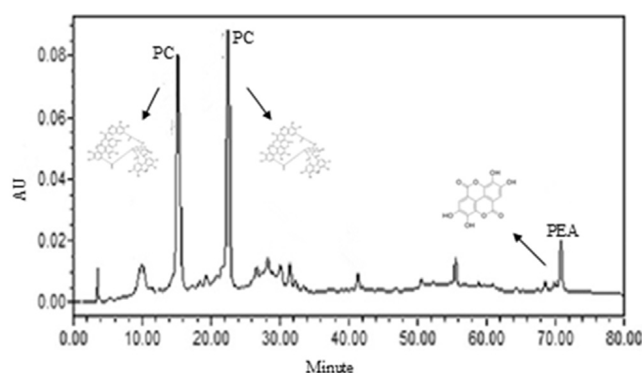

Fig. 1. HPLC of PPPs.

2.2.2. HPLC analysis of PPPs

HPLC analysis was used to identify and quantify polyphenol compounds of pomegranate peel polyphenols extracts by 1525 Waters HPLC system (Waters Corp, Milford, Massachusetts, USA) equipped with a binary pump, an UV detector (Waters 2487) and a column temperature controller. The chromatographic separation was carried out on a Zorbax SB-C18 column (4.6 mm i.d. × 250 mm, 5 μm, Agilent, American) using 1% glacial acetic acid (solvent A) and methanol (solvent B) as mobile phase at a flow rate of 1 mL/min. The gradient programme was set as follows: 0–70 min, 5–44% B; 70–80 min, 44–44% B. The chromatogram was detected at 280 nm. The phenolic extracts were prepared before injecting. The injection volume of each sample and standard solution was 20 μL and the column temperature was maintained at 30 °C.

2.3. Methods

2.3.1. Cell culture and grouping

HepG2 and normal human hepatic L-02 cells were cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (Gibco, USA), 100 mg/mL streptomycin and 100 unit/mL penicillin, and then they were incubated at 37 °C with 5% CO_2 supplied in the incubator, using the cells in logarithmic growth phase in the experiment.

Three sets of experiments were performed as following: the test substance treatment group with the final concentration of 100, 200, and 300 μg/mL of PPPs respectively, the positive control treatment group with the final concentration of 100 μg/mL of the 5-Fu, the control group. The test compounds were dissolved and diluted by the serum-free culture medium. All experiments were repeated three times independently.

2.3.2. Cell proliferation and inhibition assay

The proliferation and inhibitory effects of PPPs on HepG2 and L-02 were measured by MTT assay (Koyama et al., 2010). Cells were seeded in 96-well culture plates (5×10^3 cells/well). After 24 h, the cells were washed with fresh medium and treated with different final concentrations of the drug to each well, and each group has 5 repetitive holes and set the control group respectively. After incubating for 24 h respectively, each well is added 20 μL with 5 mg/mL of MTT, on the same condition, cells are incubated for 4 h, when the supernatant is discarded, each well is added 150 μL DMSO, shaking them up gently. Measuring the absorption ratio of the light at 570 nm with the microplate reader and calculating the survival rate of liver cancer cells after drug treatment. Cell viability was calculated by the following formula:

$$Cell\ viability\ (\%) = [(A_t - A_b)/(A_c - A_b)] \div 100\%$$

Table 1
Calibration curves and the contents of the identified polyphenols obtained from the pomegranate peel extracts by HPLC.

Component	t_R (min)	Linearity (μg/mL)	Regression equation (Y = aX + b)	R^2	Content (mg/100 mg)
Gallic acid	9.97 ± 0.09	22–110	Y = 5443X + 422.17	0.9991	2.53
Punicalagin	15.22 ± 0.09	11–500	Y = 2898X − 1121.61	0.9989	65.38
	22.49 ± 0.08				
Catechin	26.65 ± 0.05	22–110	Y = 2031X + 447.94	0.9992	12.66
Chlorogenic acid	31.53 ± 0.07	1.4–110	Y = 4728X + 156.11	0.9993	0.37
Caffeic acid	35.75 ± 0.09	0.04–80	Y = 8734X + 313.97	0.9987	0.03
Epicatechin	41.36 ± 0.04	2.2–110	Y = 1684X + 145.86	0.9994	0.99
Rutin	68.60 ± 0.06	2.8–110	Y = 1890X + 242.67	0.9990	0.34
Ellagic acid	70.86 ± 0.08	11–275	Y = 2491X − 320.39	0.9992	2.93

A_t, A_c and A_b stand for the absorbance of the test substances, the control and the blank, respectively.

2.3.3. Cell morphology analysis

Hoechst 33258 staining assay was used to visualize apoptotic cells (Liu et al., 2011). HepG2 cells were cultured in 6-well plates (4×10^5 cells/well) for 24 h and then treated with different concentrations of PPPs as the sets of experiments for 24 h. PBS was added to wash wells and the cells were added 1 mL of 4% paraformaldehyde, which need to be placed below 4 °C for l5 min. The cells were washed again by PBS and added 1 mL Hoechst33258 with the concentration of 5 μg/mL, staining for 10 min. Re-washing by PBS twice again, set the cells under a fluorescent microscope and took a picture.

2.3.4. Cell cycle analysis by the FCM

The cell cycle distribution was detected by FCM using PI (Marel et al., 2008). The cells were treated with different concentrations of PPPs and 5-Fu for 24 h. Both the suspension cells and the adherent cells were collected, then suspended in cold PBS and fixed with 70% ethanol at −20 °C overnight. Subsequently, the fixed cells were washed with cold PBS before incubation with the binding buffer containing RNase (1 mg/mL) and PI (400 μg/mL) for 30 min at 37 °C in the dark. Finally, the stained cells were analyzed by the FCM (Millipore Corporation, Billerica, MA, USA).

2.3.5. Analysis of apoptotic cells

The apoptosis were quantified by Annexin V-FITC/PI double staining assay using Annexin V-FITC detection kit (Zhou et al., 2013). After treatment with the different concentration of PPPs and 5-Fu for 24 h, all the cells were harvested, washed and resuspended in 400 μL of binding buffer. 5 μL of annexin V-FITC and 10 μL of PI were added to stain for 10 min in the dark. The apoptosis situation of the cells were detected with FCM, and then were defined as: lower left quadrant, living cells (Annexin V_/PI_); lower right quadrant, early apoptotic cells (Annexin V+/PI_); upper right quadrant, late apoptotic cells (Annexin V+/PI+); upper left quadrant, primary necrotic cells (Annexin V_/PI+).

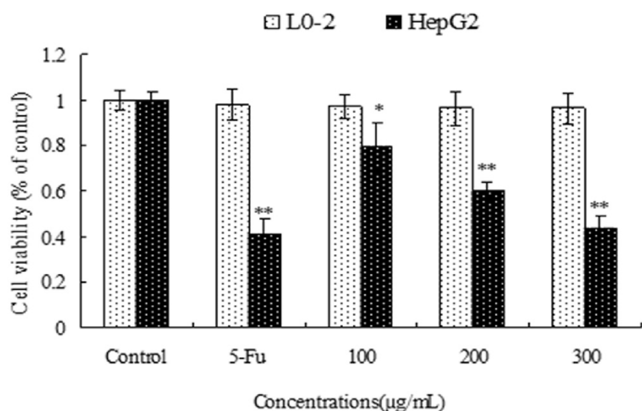

Fig. 2. Inhibitory effects of PPPs on HepG2 cells and the normal liver cells L-02 cells via apoptosis. Cells were treated with the increasing concentrations of PPPs for 24 h, and cell viability was determined by the MTT assay. Results are expressed as percentages of MTT absorbance with respect to the untreated vehicle control wells. Compared to the negative control group, *$p < 0.05$, significant difference; **$p < 0.01$, highly significant difference.

2.3.6. Measurement of intracellular ROS

The intracellular production of ROS was assessed by DCFH-DA probe (Haider et al., 2011). The cells were treated with different concentrations of PPPs and 5-Fu. After 24 h, the liquid waste is discarded to collect cells by digestion and centrifugation. Then add DCFH-DA (4 uM) into them and place them under the state of 37 °C for 30 min. Detect the generating situation of fluorescence with FCM and analyze the changes of ROS in HepG2.

2.3.7. The analysis of $\Delta\psi m$

The $\Delta\psi m$ was measured using a lipophilic cationic probe fluorochrome JC-1; JC-1 exhibits a potential dependent accumulation in mitochondria indicated by a fluorescence emission shift from 530 to 590 nm (Wu et al., 2015). Briefly, after treatment with PPPs and 5-Fu for 24 h, the cells were collected by digestion and centrifugation, take 500 μL of JC-1 resuspended cells and place in an incubator for 30 min, then the cells were collected and resuspended in the Incubation Buffer, the single-cell suspension were analyzed by FCM, the changes of $\Delta\psi m$ can be reflected by the ratio of red or green fluorescence intensity to the reaction.

2.3.8. Detection of caspase-3 and caspase-9 activities

Analysis of the activities of caspase-3 and caspase-9 were carried out by Caspase Activity Assay kit (Alfredsson et al., 2014). After the cells were treated with different concentrations of PPPs for 24 h, then collected and lysed in cold lysis buffer. After 15 min in ice, protein was obtained from supernatant by centrifugation and was mixed with detection buffer and catalytic substrate (AcDEVD-pNA and Ac-LEHD-pNA) in a 96-well plate. The mixtures were incubated for 2 h at 37 °C. Finally, the activity of caspases which was represented by the absorbance was measured at 405 nm with microplate reader.

2.3.9. Western blot for protein expression detection

Cells of control group and each group of treatment were collected and lysed in total protein extraction reagent and proteinase inhibitors. The lysates were centrifuged at 15,000 g for 30 min, supernatants were saved and their protein concentration was determined by using the Bradford assay (Li et al., 2013). After being separated by 15% SDS-PAGE, proteins were transferred to PVDF membranes and use 5% fat-free dry milk to block for 2 h. 1-

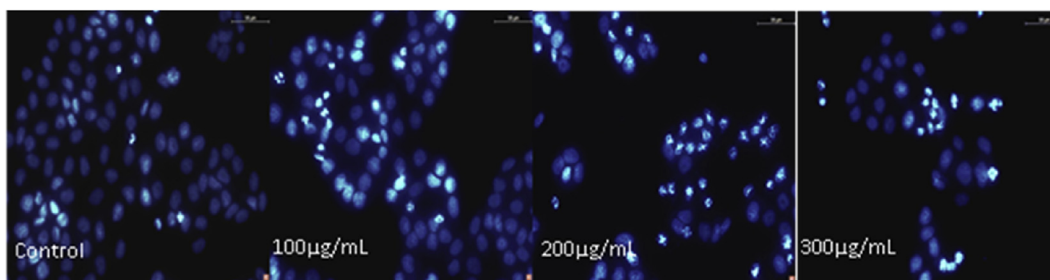

Fig. 3. Morphological changes of HepG2 treated with 100, 200 and 300 μg/mL of PPPs for 24 h.

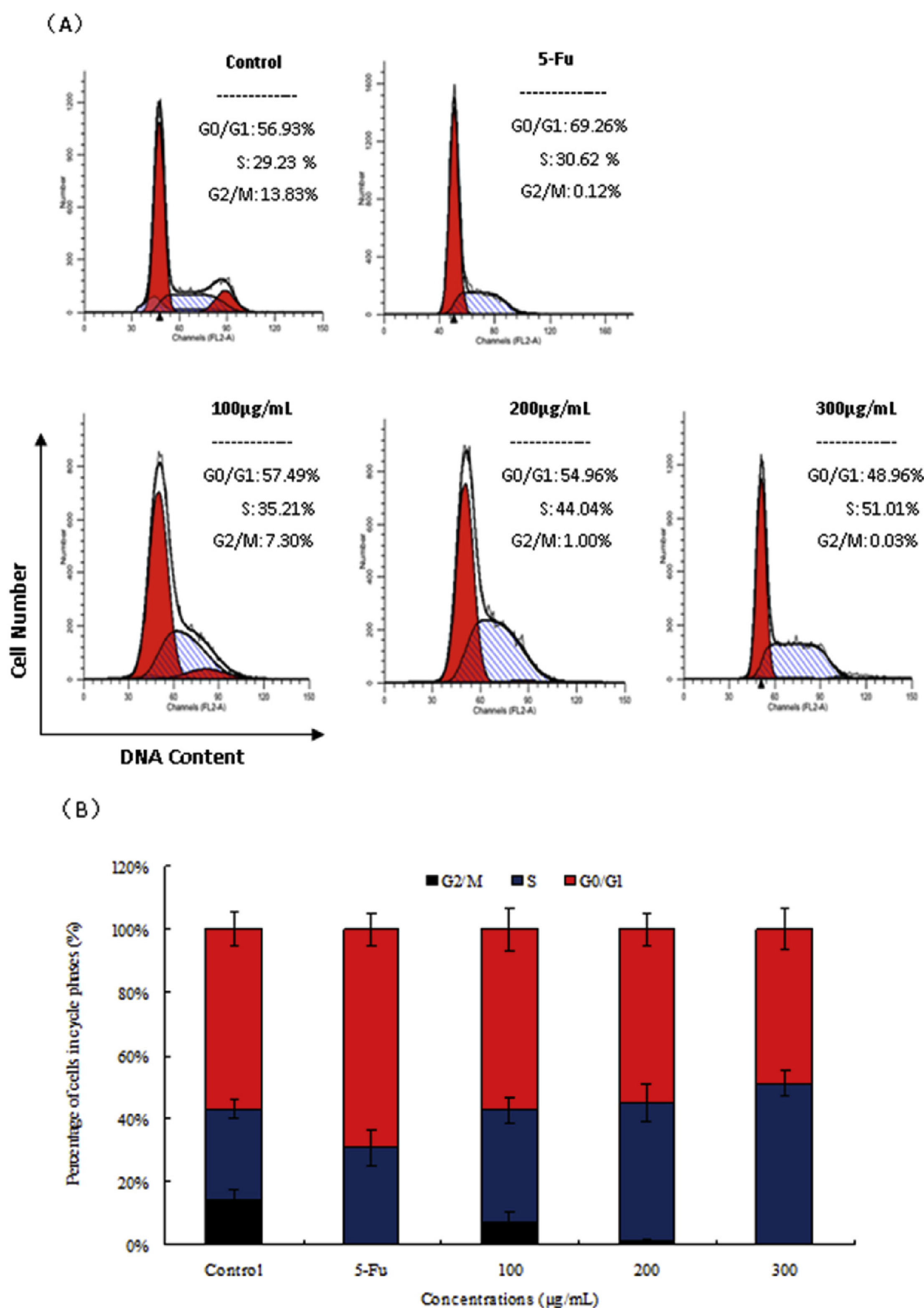

Fig. 4. Cell-cycle analysis, HepG2 cells were treated with various concentrations of PPPs and the cell phase distribution was determined by PI staining. (A) Cellular DNA content histogram; (B) the percentage chart shared by each phase of the cell.

antibody (1:500) 4 °C closed overnight, and 2-antibody (1:2000) including P53, Bcl-2, Bax, Cyt-c, PARP and β-Actin are incubated for 2 h under the condition of 37 °C. Use alkaline phosphatase color kit for coloring and take and preserve pictures. The experiment need to be repeated three times in success.

3. Result and analysis

3.1. HPLC analysis for PPPs

Polyphenols of purified pomegranate peel extracts were

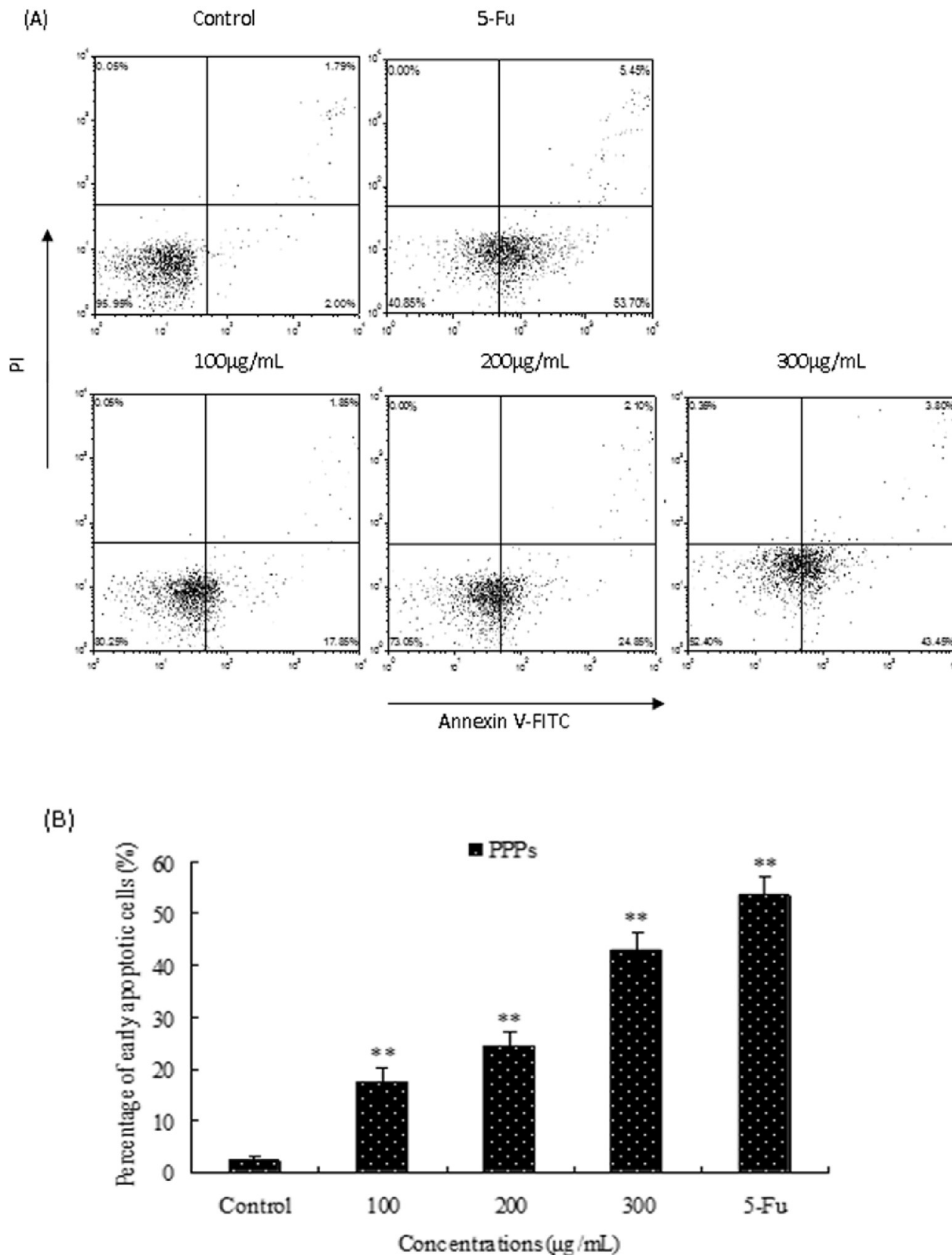

Fig. 5. The apoptotic rates of HepG2 cells induced by 100, 200 and 300 μg/mL of PPPs. (A) Cells Annexin V/PI staining bitmap; (B) The histogram depicted the early apoptotic rate of HepG2 cells, **p < 0.01, highly significant difference.

determined by HPLC analysis, monitoring the chromatogram at 280 nm (Fig. 1). HPLC analysis by direct infusion of peel extracts demonstrated the presence of 7 compounds was shown in Table 1. Polyphenol compounds were identified in turn as follows: allic acid (9.97 min), punicalagin with two peaks (Isomers, 15.22 and 22.49 min), catechin (26.65 min), chlorogenic acid (31.53 min), caffeic acid (35.75 min), epicatechin (41.36 min), rutin (68.60 min) and ellagic acid (70.86 min).

The total phenols content of PPPs was 87.81%. The punicalagin is a major component of PPPs which recorded 65.38 mg/100 mg, and the other phenols contents of catechin, ellagic acid, gallic acid, epicatechin, chlorogenic acid, rutin and caffeic acid separately were 12.66, 2.93, 2.53, 0.99, 0.37, 0.34 and 0.03 mg/100 mg, respectively.

3.2. The effect of PPPs on viability of HepG2 cells

As shown in Fig. 2, PPPs inhibited the survival rate of HepG2 cells in a dose-dependent manner, but had no significant effect on L-02 cells. The inhibitory rate of HepG2 cells treated with PPPs at 100, 200 and 300 μg/mL for 24 h was 16.4%, 36.3%, 54.2% respectively, and the inhibitory effect of 300 μg/mL PPPs nears the positive control of 5-Fu.

3.3. Effects of PPPs on morphology of HepG2 cells

The cells morphology texted by Hoechst33258 staining was shown in Fig. 3. Compared to the control group, cells of treatment group were deformed and deeply stained. The cells of shape

changed significantly, which are characteristics as the nuclear shrinkage and chromatin margination. With the increasing of PPPs concentration, the number of cells was decreased, and the nuclear condensation and nuclear fragmentation phenomenon were strengthened.

3.4. Effect of PPPs on HepG2 cell cycle arrest

The influence of PPPs on HepG2 cell cycle was shown in Fig. 4(A, B). After the different concentrations of PPPs acting on HepG2 cells for 24 h, with the increasing of PPPs concentration, the HepG2 cells in S-phase are increased, the percentage of G2/M phase is declined, the DNA histogram results of cell cycle analysis indicated that the number of cells in the S phase was enhanced from 29.23% to 51.01% ($p < 0.01$), accompanied a decrease of the percentage of cells in the G2/M phase from 13.83% to 0.03%. The results indicated that PPPs could arrest the HepG2 cell cycle in S phase.

3.5. Effect of PPPs on the apoptosis rate of HepG2

The results of Annexin V-FITC and PI double staining and flow cytometry analysis were shown in Fig. 5. The cells being treated in different concentrations of PPPs for 24 h, the decaying rate of the cell in treated group obviously higher than that in the reference group. In contrast with control group, the early apoptosis rates of cells was 17.85%, 24.85%, 43.45% ($P < 0.01$) after treatment with different concentrations of PPPs, respectively. Moreover, PPPs caused the variation of late apoptosis rates, although the change is weak. The results suggested that PPPs induced cell apoptosis in a dose-dependent manner.

3.6. ROS production of HepG2 cells

The results of PPPs on the role of ROS level in HepG2 cell were shown in Fig. 6. Compared with the 0.12% H_2O_2 of the control group, the levels of H_2O_2 in PPPs groups increased to 40.79%, 54.58% and 64.53% ($P < 0.01$), respectively. PPPs promoted the H_2O_2 production dose-dependently. The figures indicated that PPPs can promote the increase of ROS level leading to the impairment and the apoptosis flowingly. Thereby, the level of ROS was higher than basal values, and the effect of PPPs was close to the content of H_2O_2 of 5-Fu in control group.

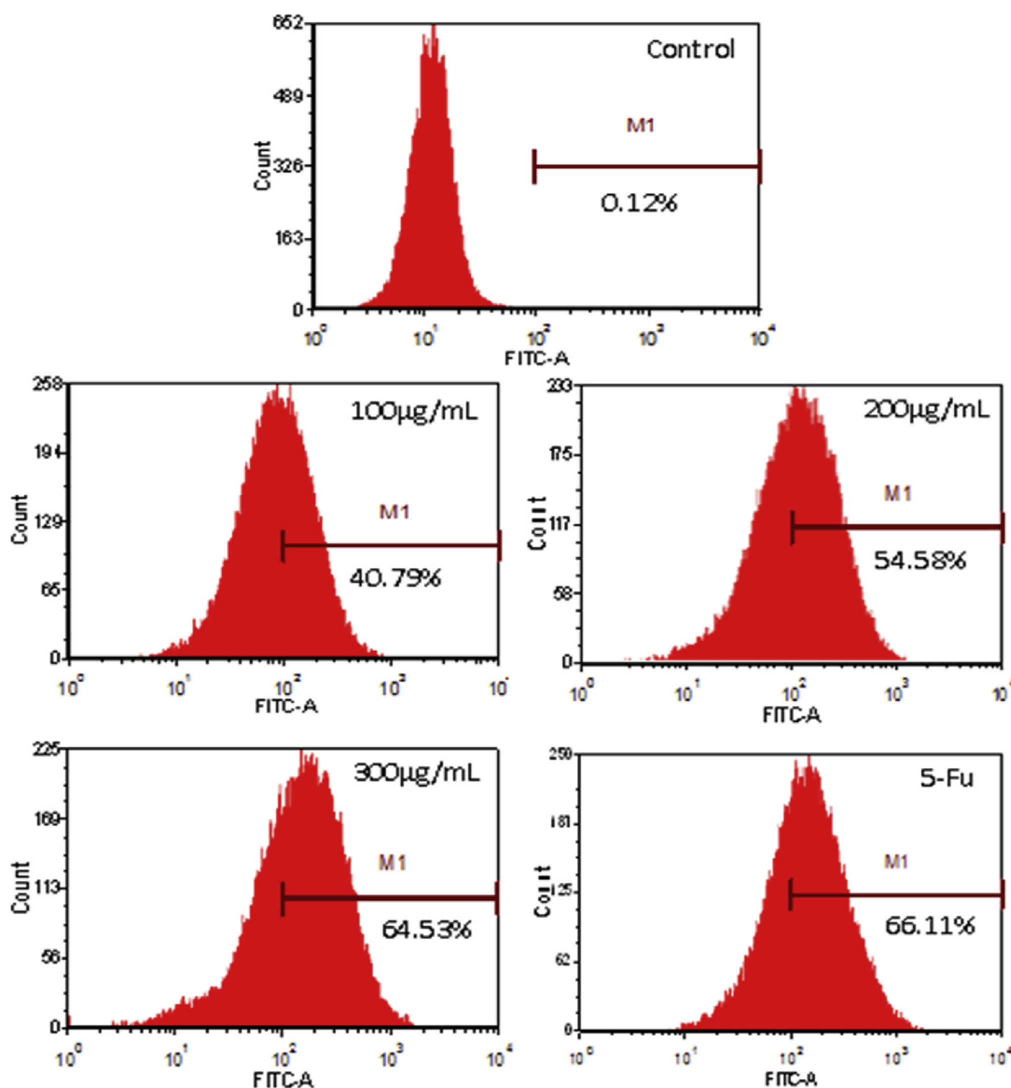

Fig. 6. H_2O_2 levels changes of HepG2 treated with 100, 200 and 300 μg/mL of PPPs for 24 h.

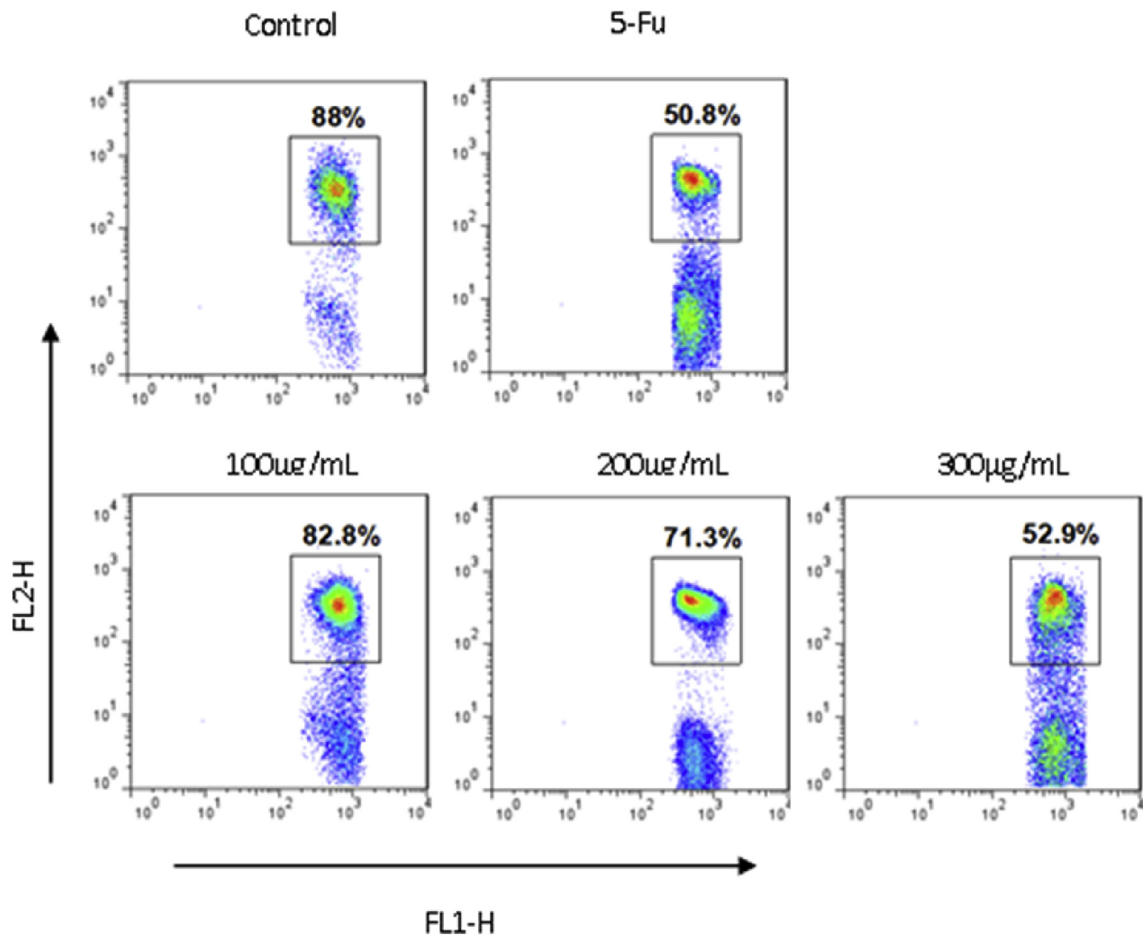

Fig. 7. Effects of different concentrations PPPs on the Δψm of HepG2 cells.

3.7. The result of the measurement of Δψm

The results of the Δψm were shown in Fig. 7. Compared with the control group, with the concentration of PPPs increasing, the green fluorescence in treatment group were enhanced from 17.2% to 47.1% ($p < 0.01$). The results indicated that PPPs could cause a significant decrease in the mitochondrial Δψm.

3.8. Caspase-3 and caspase-9 activity results

The results of caspase-3 and caspase-9 activities were shown in Fig. 8. Compared to the negative control, the activities of caspase-9 in the cells of treatment groups were increased by 1.5-fold, 1.8-fold, 2.2-fold ($P < 0.01$) respectively after PPPs treatment. Similarly, the intracellular caspase-3 activity was increased by 1.3-fold, 1.6-fold, 2.0-fold ($P < 0.01$) respectively, It showed that PPPs could induced apoptosis in HepG2.

3.9. Western blot

The results of Western Blot were shown in Fig. 9. Compared with the control group, p53 protein expression was significantly increased by PPPs, while the expression of Bcl-2 that suppress apoptosis of protein and Bax for promoting decreased. But the ratio of Bax/Bcl-2 was significantly increased, and the expression of Cyt-C was also significantly increased, meanwhile, the expression of PARP protein was dramatically decreased. This series of changes in protein expression indicates that PPPs could induce apoptosis of HepG2 cells via the intrinsic mitochondrial pathway regulation.

4. Conclusion

This study show that PPPs could significantly inhibit HepG2 cell proliferation, induce apoptosis and arrest the cells cycle in HepG2 cells with a dose-dependent manner. The effect of 300 μg/mL PPPs is close to the positive control. These changes indicate that PPPs

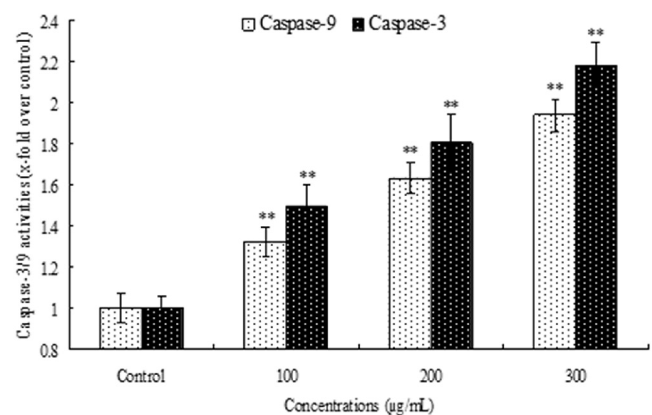

Fig. 8. Effects of Apoptosis-related proteins Caspase3/9 activities, compared with the negative control group, $^{*}p < 0.05$, significant difference; $^{**}p < 0.01$, highly significant difference.

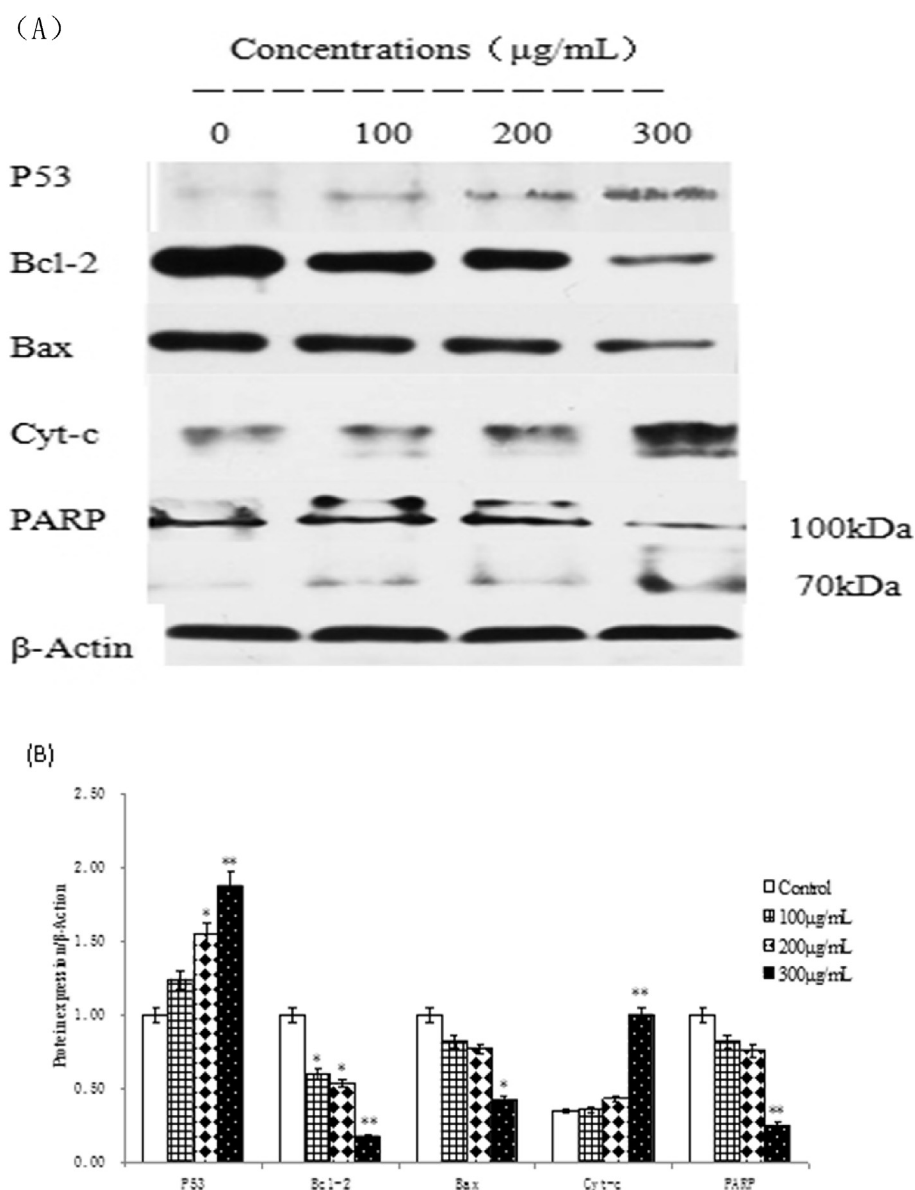

Fig. 9. Effects of apoptosis-related proteins in response to PPPs treatment were assessed by western blot analysis. (A) Images shown are typical western blot bands for Cyt-c, PARP, p53, Bcl-2 and Bax, (B) statistical analysis. Compared to the negative control group, *$p < 0.05$, significant difference; **$p < 0.01$, highly significant difference.

could induce apoptosis of HepG2 cells via the intrinsic mitochondrial pathway regulation.

5. Discussion

Nowadays, chemotherapy is the main measures of cancer therapy, which uses medicine to weaken and destroy cancer cells in the body. However, apoptosis is a slow process of death in cells which is triggered by receiving some signals according to certain procedures under some physiological or pathological conditions. It makes the unwanted cells die and the number of organ tissue cells by this way to maintain a constant balance (Ding et al., 2009). At the same time, it is important to control cell proliferation and prevent the growth of tumors (Lansky and Newman, 2007). Currently, much effort has been made to develop new drugs, which are not only significantly induced tumor cells apoptosis, but also have a relatively low possibility of side effects. The natural compounds from plants reported to have anticancer effect has been studied extensively (Gao et al., 2014).

In recent years, with the deep pharmacological studies of pomegranate, we found that pomegranate has extensive medicinal values (Shaban et al., 2013). The products extracted from pomegranate seed can increase immune function of B cells. The pomegranate seed oil can inhibit the formation of Colon cancer induced by the chemical substances. The products extracted from pomegranate peel and seed can inhibit the growth of prostate cancer cells (Mee Young Hong et al., 2008) etc. Plant polyphenols have strong antioxidant, anti-cancer, anti-tumor, anti-aging and inhibiting cholesterol increase and other complications activities (Tariq Ismail et al., 2012). Polyphenols can prevent the outbreak of cancer and suppress the different stages of cancer. The polyphenols could inhibit the proliferation of hepatocytes. But the related mechanism of action has not been reported clearly (Sudeshna Mukherjee et al., 2013). So this experiment studied the effect of PPPs on proliferation and apoptosis of HepG2 cell. This article vividly illustrated that the molecular mechanism of the PPPs induce apoptosis of HepG2 cell,

we clearly know PPPs could cause the stop of cell cycle so as to inhibit the proliferation of HepG2, and the mechanism of the PPPs inhibiting the proliferation of HepG2 is about the participation in mitochondrial apoptotic pathways of cells.

The study indicates that PPPs can inhibit the cancer cell and induce apoptosis of cancer cells. The mechanism is regulated by the intrinsic mitochondrial pathway to arrest the cell cycle and induce apoptosis of HepG2 cell. This research provides the theoretical basis for the further study of polyphenols, and PPPs play a supportive role in natural agents to the treatment and prevention of human liver cancer and health care.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.04.020.

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