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Original Article

Mulberry fruits extracts induce apoptosis and autophagy of liver cancer cell and prevent hepatocarcinogenesis in vivo

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ABSTRACT

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Taiwan. Many risks factors induce liver chronic inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma. Mulberry fruits containing polyphenols to remove free radicals and mitigate inflammation has been reported to not only against gastric cancer, melanoma and leukemia but also prevent liver injury induced by alcohol or CCl_4 in previous researches. The aim of this study is to examine whether Mulberry could inhibit hepatocarcinogenesis. In animal experiment, diethylnitrosamine (DEN) was used to induce hepatic tumorgenesis. After injecting DEN, the rats treated with mulberry water extracts (MWE) had less and smaller tumor than others without MWE. Moreover, MWE reduced the serum ALT and AST, HCC marker, cleavage caspases, Ser-15-p53 and Ser46-p53 induced by DEN. Further, we observed that mulberry polyphenol extracts (MPE) inhibited the cell growth of HepG2 cell and Hep3B cell. By using flow cytometry and western blotting methods, MPE induced HepG2 cell apoptosis by increase subG1 cells and the elevated expression of caspase-3/8/9. Instead of apoptosis, MPE caused Hep3B cells autophagy by inhibiting Akt and mTOR phosphorylation. Comprehensively, mulberry extracts has a potential to be a health supplement to prevent hepatocarcinogenesis in the future.

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1. Introduction

Liver cancer is the sixth most prevalent cancer globally, and hepatocellular carcinoma (HCC) is the most frequent and fatal type of liver cancer that have very low survival rate [1]. Risk factors including chronic HBV (hepatitis B virus) and HCV (hepatitis C virus) infections, autoimmune hepatitis, chronic alcohol use, obesity and diabetes mellitus cause HCC progression [2]. The molecular basis of HCC progression originated in mature liver cells or progenitor cell may differ depending on diverse mechanism. Loss of cell cycle and senescene control, dysregulation of apoptosis, and liver inflammation triggered by cytokines and NF-κB pathway are important mechanisms associated with the HCC [3]. Many risk factors and different mechanisms increase the difficulty on the therapy of HCC.

Common treatment strategies for HCC include chemotherapy, loco regional ablation, surgical resection, intervene therapy or liver transplantation [4]. Only early-stage tumors can be cured, however HCC often diagnosed at an advanced stage. Sorafenib or Nexavar, a MAP Kinase inhibitor, is the first-line drug that has been approved for treatment of end stage patients with advanced or metastatic HCC [5]. Unfortunately, patients on Sorafenib generally suffer from the drug side effects such as diarrhea, hand-foot skin disease, fatigue, anorexia and alopecia. Therefore, how to reduce the incidence of liver cancer may be a better strategy.

Polyphenols including phenolic acids, flavonoids, anthocyanins and polyphenolic amides are the biggest group of phytochemicals [6]. Recently, many polyphenols from a wide range of foods and herbs are reported to exert protective and therapeutic effects on liver diseases via complicated mechanisms [7]. Mulberry belongs to the genus *Morus* of the family Moraceae. Analysis of Mulberry water extracts (MWE) reveals the high composition of polyphenols (MPE) containing phenolic acids (5.12%), flavonoids (8.23%) and anthocyanins (5.61%) [8]. In our previous studies, anthocyanin-rich Mulberry extracts (MAC) suppressed gastric cancer cell survival and tumorigenesis, and induced apoptotic death *in vitro* and in vivo by targeting p38/p53 and the c-jun pathways [9]. MAC could mediate B16-F1 cell metastasis by reduction of matrix metalloproteinase-2 and -9 activities involving the suppression of the Ras/PI3K signaling pathway [10]. The hepatoprotection of Mulberry extracts was also proved. Our previous studies showed MWE could reduce lipid accumulation, suppress fatty acid synthesis, and stimulate fatty acid oxidation via AMPK pathway [11]. MWE exhibited protective and curative effects against CCl_4-induced liver damage and fibrosis via decreasing lipid peroxidation and inhibiting proinflammatory gene expression [12]. In addition, MWE offered the hepatoprotective effects on acute liver failure induced by lipopolysaccharides [13]. Yang et al. revealed that Mulberry leaf polyphenol extracts mediated the cell death of hepatocellular carcinoma cells and the p53 might play an important role in regulating the death mechanism [14]. Recently, we further found that Mulberry leaf extract targeted the proliferation signal pathway of the inflammatory response of adipocytes in HCC and could be to prevent obesity-mediated liver cancer [15].

We aimed to clarify the effect of MWE on reducing liver carcinogenesis. In this study, we found that MWE inhibited DEN-induced HCC formation. Then MPE, the major composition of Mulberry phytochemical, was used to clarify the related mechanisms. By using p53-positive HepG2 cells and p53-negative Hep3B cells, MPE induced HCC cells death through different way, indicating the p53 status played a critical role on HCC.

2. Materials and methods

2.1. MWE and MPE preparation

The 100 g of dried Morus alba L. (mulberry) fruit was stirred with water (1000 mL) and then centrifuged (8000 g) for 10 min. The supernatant was then collected and lyophilized (−80 °C for 12 h) to obtain the MWE and stored at −20 °C. On the other hand, the 100 g dried fruit was suspended in 500 mL methanol and stirred with a magnetic stirring-bar for 3 h at 50 °C in a water bath. The extract was filtered and lyophilized under reduced pressure at room temperature. The powder was dissolved in 500 mL of 50 °C distilled water, followed by extraction with 180 mL of ethyl acetate three times. The extraction was collected and lyophilized under reduced pressure at room temperature again. The powder of MWE and MPE were dissolved in distilled water and ethanol (EtOH) respectively, stored at −80 °C overnight, and lyophilized.

2.2. Animals and treatment

The rat license of the laboratory (No. 1204) was issued by the CSMU. All procedures involving animals were approved by the guidelines of IACUC (Institutional Animal Care and Use Committee) of CSMU (Animal Center of Chung Shan Medical University). 4–5 weeks of age male Wistar rats (BioLasco Taiwan, Taiwan) were used. The rats were allowed to acclimate for at least 7 days on a standard laboratory diet under environmentally controlled conditions (22 ± 2 °C and 55% ± 5% relative humidity, and 12 h light/dark cycle) with free access to food and water. According to a suitable pharmacological dose of MWE for humans in a daily diet, 30 rats were randomly divided into 5 groups: control (normal saline-treated), 2% MWE-treated, DEN-treated (40 mg/kg), DEN-treated +1%

MWE, and DMN-treated +2% MWE groups for 4 months. DEN was dissolved in normal saline and then intraperitoneally (i.p.) administered to rats per week at doses of 40 mg/mL/kg. Meanwhile, the rats in the treatment groups were administered orally by normal diet with 1% or 2% MWE daily for 4 months.

2.3. Serum biochemical

The body weight and blood samples of rats were recorded and collected every month. Blood samples were centrifuged at 3000 g for 20 min to obtain the serum. Plasma-lipid concentrations were determined using commercially available kits. Alanine amino transferase (ALT), aspartate amino transferase (AST), were measured using Beckman Coulter AU680 chemistry analyzer (Beckman Coulter, Inc. Brea, CA, USA).

2.4. Histopathological evaluation of hepatic tumors

Liver parts were collected from the sacrificed and immersed in neutral buffered formalin 10% for at least 24 h. Fixed tissues were processed routinely for paraffin embedding, and 4-μm sections were stained with Hematoxylin and Eosin (H&E) for general histological architecture. Stained areas were viewed using an optical microscope with a magnifying power of 40X.

2.5. Immunohistochemical analysis in hepatic tissue

Immunohistochemical staining for anti-alpha fetoprotein (AFP) (Mouse, Sc-51506, Santa Cruz, 1:200) was performed according to manufacturer instructions. Briefly, the slides were deparaffinized in xylene, rehydrated in a graded alcohol series and blocked with 3% H_2O_2 or 5% BSA in methanol for 10–30 min. After, the slides were washed with PBS and then immunostained with primary antibodies for AFP. Next, overnight incubation with the primary antibody was conducted. After removing the antibody, the complement was placed, and the HRP conjugate (Advance HRP Polymer) was applied for 30 min. After washing, sections were counterstained with Meyer's hematoxylin and washed with tap water.

2.6. Cell line and cytotoxicity assay

The liver cancer cell lines HepG2 and Hep3B were purchased from the Bioresource Collection and Research Center. The cells were cultured in minimum essential medium (HyClone; GE Healthcare Life Sciences) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific Inc.) at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was measured by thiazolyl blue tetrazolium bromide (MTT) assay. HepG2 and Hep3B cells were placed in a 24-well plate at a density of 5×10^4 cells/mL respectively, and treated with MPE at different concentrations (0, 0.125, 0.25, 0.5, and 1 mg/mL) or solvent control (alcohol; EtOH) for 1, 2, and 3 days. Then, the medium was changed and incubated with MTT solution (0.5 mg/mL)/well for 3 h. The medium was removed, and formazan was solubilized in isopropanol and measured spectrophotometrically at 563 nm using microplate reader.

2.7. Cell cycle analysis

HepG2 and Hep3B cells were seeded in 10 cm dishes, treated with MPEs (1.0 mg/mL) alone for 24 h, harvested with trypsin–EDTA, washed with cold PBS, fixed with cold 70% alcohol for 4 h at −20 °C, removed from the alcohol, and stained with 50 μg/mL propidium iodide solution containing 10 μg/mL RNase A, in darkness for at least 30 min on ice. The distribution of cells in different cell cycle phases was determined using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA). A minimum of 10,000 cells per sample was counted and DNA histograms were analyzed using Cell Quest software (BD Biosciences) to calculate the percentage of cells in each peak.

2.8. Determination and quantification of apoptosis

Cells (1×10^6 cells) cultured in 6 cm dish were treated with different concentrations (0, 0.25, 0.5, and 1 mg/mL) of MPE for 24 h, while adding EtOH for the control. Cells in each concentration and control were fixed with ice-cold 4% paraformaldehyde in PBS solution for 30 min, washed with PBS and then stained with 1 μg/mL DAPI for 15 min or incubated with the TUNEL stain provided by the manufacturer. The cells were photographed under a fluorescence microscopy (LAS-4000 Fujifilm, Japan).

2.9. Western immunoblotting

Caspase-3, 8, 9, Bax, Bcl-2, Fas, Fas-L, PI3K, p-mTOR, and cell cycle related proteins were purchased from Santa Cruz Biotechnology (Dallas, USA). P53, phospho-p53 ser15, phospho-p53 ser46, Akt, p-Akt, p-AMPK, and puma proteins were purchased from Cell Signaling Tech. (Beverly, MA). LC3 II was purchased from Abcam company (Cambridge, UK). The cells were collected and rinsed with PBS at room temperature. Then 0.5 mL of cold RIPA buffers (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, 150 mM NaCl, pH 7.5) with fresh protease inhibitor was added. Liver tissues were homogenized in lysis buffer. Proteins were collected by centrifugation at 12,000 *g* and 4 °C. The total protein samples were run on 8–12% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes and incubated in blocking buffer (5% milk and 0.5% bovine serum albumin) for 1 h at room temperature and then incubated overnight at 4 °C with the primary antibodies. After three washes with Tris-buffered saline containing 0.05% Tween 20 (TBST), membranes were incubated with secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Antigen-antibody complexes were then developed with an electrochemiluminescence (ECL) kit (Millipore) and analyzed using AlphaImager Series 2200 software (Alpha Innotech, San Leandro, CA, USA).

2.10. Acridine orange (AO) staining

AO staining was used to detect the presence of acidic vesicular organelles (AVOs) after MPE treatment. HepG2 and Hep3B cells (5×10^5 cells/well) were treated with MPE or EtOH (control group) for 24 h, then cells were washed twice in cold PBS and

fixed with 4% formaldehyde for 10 min. Following, cells were stained with 5 μg/mL AO solution for 15 min at 37 °C in the dark and observed using an inverted fluorescence microscope ((LAS-4000 Fujifilm, Japan). The uptake of acridine orange was calculated by subtracting mean fluorescent intensity of untreated control cells (autofluorescence).

2.11. Statistical analysis

Data were expressed as means ± SD from at least three independent experiments. The difference among the three groups was analyzed by one-way ANOVA. The difference between two groups was analyzed by Student's *t*-test performed with Sigmaplot software (version 12). Statistical significance was detected at the 0.05 level.

3. Results

3.1. MWE treatment reduced DEN-induced liver tumorigenesis

Transaminase enzymes are known as important markers of hepatocellular damage. As shown in Table 1, following DEN treatment for two months, the activity of serum ALT and AST was elevated in comparison with the control rats. DEN treatment for four months caused a significant ($p < 0.05$) increase in the levels of ALT and AST. Supplementation of rats with the MWE attenuated the liver transaminase from rising. Gamma-glutamyltransferase (γ-GT) is a membrane-bound enzyme involved in the glutathione (GSH) metabolism. Elevated γ-GT expression had been observed in alcohol consumption, acute and chronic liver disease and oxidative stress, and various tumors [16]. In this study, a significant increase in the activity of γ-GT was observed in the DEN-induced group for four months, whereas in MWE and DEN-treated animals the enzyme activities were reversed.

Animals were sacrificed after four months treatment, the livers and body weight, and tumor foci were measured. Lower body weight and higher liver weight were observed in DEN-induced group (Table 1), tumor foci measuring between 1.0 and 4.5 mm in diameter could be detected in all of the DEN-induced rats (Fig. 1A). Notably, in the DEN-induced rats, there was a significant delay in liver carcinogenesis after receiving MWE for 4 months (Table 1, Fig. 1A and B). Alpha fetoprotein (AFP) has been recognized as a key regulator of cell proliferation in HCC [17]. As shown in Figs. 1C and 2A, IHC staining and immunoblotting assay demonstrated that MWE could reduce the AFP expression induced by DEN. Various etiological factors such as drugs, viruses, alcohol, and cholestasis cause the hepatic apoptosis [18]. Cleavage caspases are the hallmark of apoptosis and are determined by immunoblotting assay. DEN induced the expression of cleavage caspase-3, 8, 9 and LC3 II in normal tissue whereas MWE treatment altered the increased levels of these proteins in DEN-induced group, indicating that apoptosis was occurred in damaged liver cells (Fig. 2B). Following, we investigated the p53 expression in damaged liver cells induced by DEN and found that MWE lessened the mighty increase of p53 and ser46-p53 were observed in DEN-induced group. On the other hand, MWE raised the expression of cleavage caspase-3, 8, 9 and LC3 II in tumor foci in DEN-induced groups (Fig. 2C). The results indicated that MWE may protect liver damage and HCC formation from DEN via p53-dependent and -independent cell death pathways.

3.2. MPE was cytotoxic to both HepG2 and Hep3B cells

To investigate the effects of *Mulberry* extracts on cell viability in HepG2 cell (wild type p53) and Hep3B cell (p53 null), the cells were treated with 0, 0.125, 0.25, 0.5, and 1 mg/mL MPE for 24–72 h and cell proliferation was assessed using the MTT assay. MPE decreased the viability of HepG2 cells in a time- and dose-dependent manner (Fig. 3A). Following treatment

Table 1 – The serum value of ALT and AST were decreased by MWE after treatment with DEN for two and three month.

Second Month	Control	MWE	DEN	DEN + 1% MWE	DEN + 2% MWE
ALT (U/L)	91.60 ± 9.22	84.00 ± 4.80	101.20 ± 8.87	98.50 ± 3.47	94.00 ± 3.17
AST (U/L)	46.00 ± 4.66	34.40 ± 2.40	55.00 ± 6.30	52.33 ± 6.98	52.16 ± 3.16
Body weight(g)	431.33 ± 8.38	440.83 ± 7.52	397.60 ± 6.59	373.00 ± 7.10	368.60 ± 8.30
Third Month					
ALT (U/L)	92.80 ± 4.39	92.00 ± 3.30	124.15 ± 7.44†	114.20 ± 5.52	111.17 ± 1.20
AST (U/L)	62.00 ± 2.46	56.80 ± 4.14	87.80 ± 6.71†	85.40 ± 4.29	79.75 ± 4.85
Body weight(g)	513.83 ± 11.70	527.17 ± 10.40	463.00 ± 9.82	431.20 ± 9.04	444.00 ± 12.77
Forth Month					
ALT (U/L)	104.00 ± 8.12	89.40 ± 6.48	155.75 ± 10.79†	118.00 ± 7.67*	133.00 ± 7.67
AST (U/L)	66.16 ± 3.07	68.50 ± 9.60	116.33 ± 12.46†	76.89 ± 3.80*	77.00 ± 7.67*
R-GT (U/L)	5.33 ± 0.80	4.25 ± 1.03	42.85 ± 10.92†	18.20 ± 2.39*	17.00 ± 5.02*
Body weight(g)	576.17 ± 8.83	587.1 7 ± 11.29	471.17 ± 10.16	501.80 ± 12.73	499.88 ± 19.47
Liver weight(g)	17.33 ± 0.85	19.43 ± 0.77	24.21 ± 3.59	23.51 ± 2.95	21.26 ± 1.34
Liver weight/Body weight ratio* 100%	3.1076% ± 0.17	3.3055% ± 0.08	5.1147% ± 0.75	4.6703% ± 0.56	4.1995% ± 0.33
Tumor foci	0	0	23.85 ± 5.11†	11.50 ± 2.67*	9.71 ± 2.21*

Control: normal rats; **MWE**: rats were fed with 2% MWE; **DEN**: rats were injected with DEN: **DEN + 1% MWE**: rats were injected with DEN and 1% MWE diet; **DEN + 2% MWE**: rats were injected with DEN and 2% MWE diet.
†$P < 0.05$: significant difference compared with the control group determined by Student's f test.
*$P < 0.05$ was compared with DEN group.

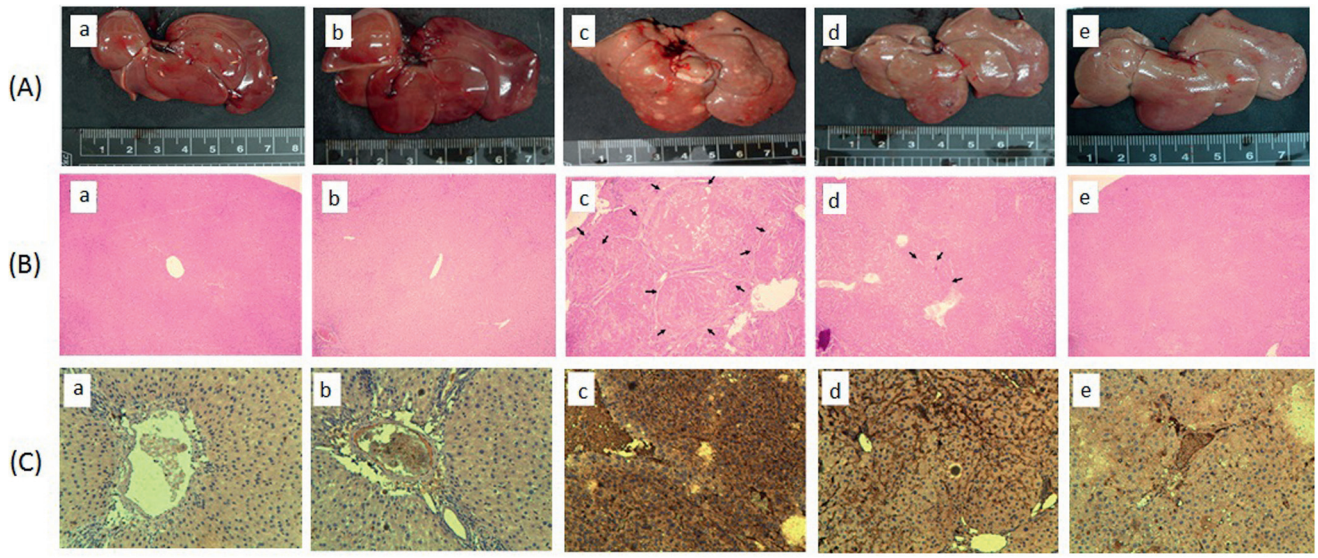


Fig. 1 – MWE reduced the DEN-induced liver tumors. Rats were injected DEN and fed with MWE (1%, 2%) for four months. (A) Rats were sacrificed and liver was taken photograph. (B) Paraffin-embedded sections of liver from rats were stained with hematoxylin and eosin. Representative photomicrographs are shown as 40X. (C) AFP expression of liver from rats were assayed by IHC (100X). [(a) Control rats; (b) MWE rat; (c) DEN rats; (d) DEN + 1% MWE; (e) DEN + 2% MWE.].

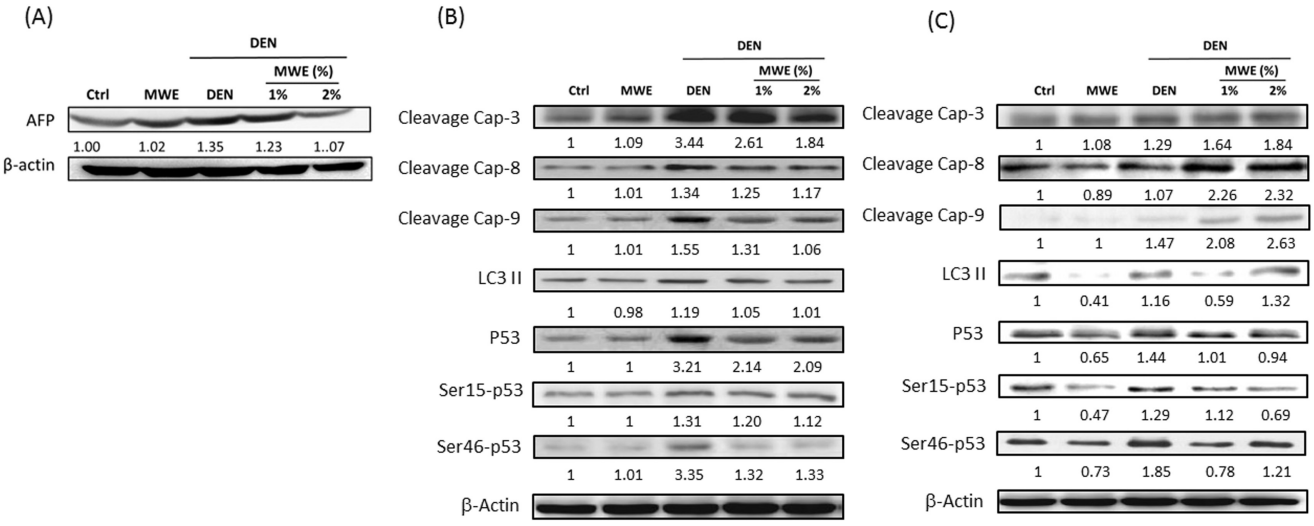


Fig. 2 – Effect of MWE on HCC marker and apoptosis protein expression in DEN-treated rats. (A) After four months treatment with DEN and MWE (1%, 2%), liver proteins were subjected to SDS-PAGE followed by Western blot to determine the expression of AFP. The levels of caspase-3, caspase-8, caspase-9, LC3 II, p53, ser15-p53 and ser46-p53 from normal tissue (B) and tumor foci (C) from liver were also detected. The results were represented by using an ECL system. β-actin was the loading control.

with 0.25 and 0.5, and 1 mg/mL MPE, the viability of HepG2 cells was significantly decreased at 24, 48 and 72 h compared with the untreated negative control group. Similarly, 0.25 and 0.5, and 1 mg/mL MPE significantly decreased the viability of Hep3B cells at 24, 48 and 72 h (Fig. 3B).

3.3. *MPE promoted the HepG2 cell death via apoptosis and PI3K/Akt pathways*

The impact of MPE on the distribution of HepG2 and Hep3B in different phases of the cell cycle was next examined. We found that the percentage of HepG2 at G0/G1 phase increased in a dose-dependent manner causing cell cycle arrest (Fig. 4A). At 1 mg/mL, there was a marked increase in subG1 phase cells. DAPI staining indicated that MPE was able to induce apoptosis in HepG2 cells (Fig. 4B). At 0.5 and 1 mg/mL of MPE, apoptotic cells were notably visible. However, various concentrations of MPE neither affected the Hep3B cell cycle distribution nor induced apoptosis in Hep3B cells. These results revealed MPE triggered HepG2 cell apoptosis whereas MPE induced Hep3B cell death via other pathway.

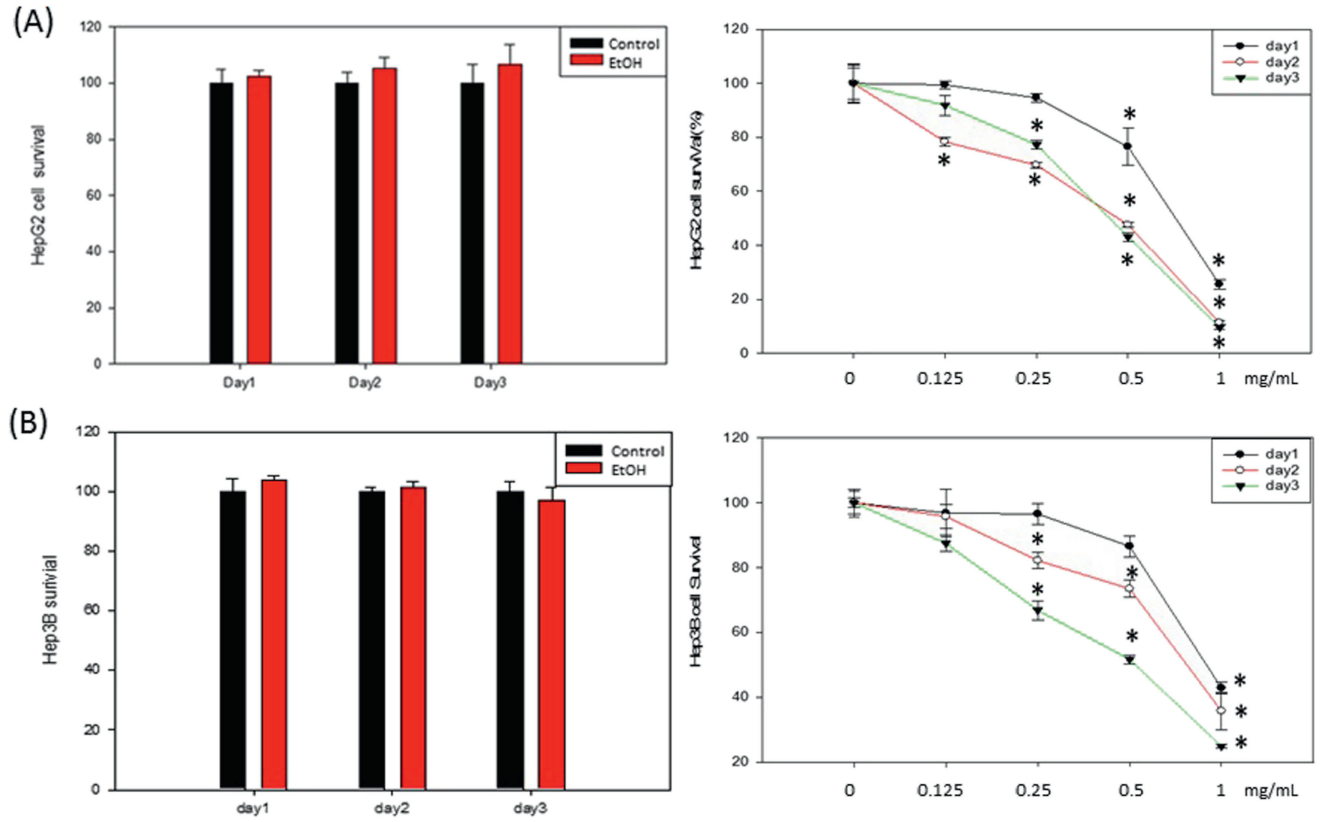


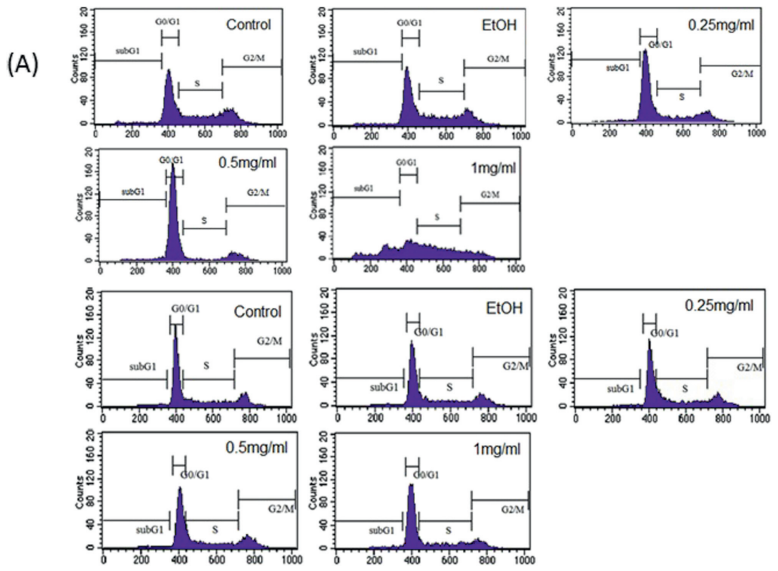
Fig. 3 – The effects of *MPE* on cell viability in HepG2 cell and Hep3B cell. HepG2 (A) and Hep3B (B) cells were treated with or without MPE under different concentrations for 24–72 h. Cell viability was measured by MTT assay. The results were shown as the means ± SD of four independent experiments. EtOH was solvent control group. *$p < 0.05$ was compared with control.

The expression levels of Bcl-2 family and caspases proteins were determined to confirm if MPE induced-apoptosis followed the intrinsic apoptotic pathway. It was observed that the expression of Bax increased and Bcl-2 decreased following the treatment with MPE, which may have resulted in apoptosis (Fig. 5A). Compared with the untreated control cells, MPE-treated HepG2 cells demonstrated a raising cleavage cap-3 and -9 expression. We next explored whether extrinsic related proteins mediated the MPE-induced apoptosis in HepG2 cells. As shown in Fig. 5B, the protein expression levels of Fas, Fas-L and cleavage Caspase-8 also went up. These results indicate that MPE triggered HepG2 apoptosis was involved in intrinsic and extrinsic caspase pathways.

p53 phosphorylation has been widely investigated and is associated with its stabilization. Notably, Ser-15 phosphorylation is known to be critical for p53-dependent transactivation [19]. p53 phosphorylation at Ser-46 leads to p53-target gene transcription and the activation of p53-dependent apoptosis pathway [20]. Because 0.25 and 0.5 mg/mL MPE treatment of HepG2 cells resulted in G0/G1 phase arrest and 1 mg/mL MPE led cell apoptosis, we examined the effect of MPE on p53 phosphorylation and cell cycle-regulatory molecules. Exposure of cells to 0.25 and 0.5 mg/mL MPE enhanced the phosphorylation of p53 on Ser15, and phosphorylation on serine residues 46 was induced by 1 mg/mL MPE (Fig. 5C). MPE treatment also increased in cells' levels of p53 downstream target, Puma, p21 and p27, and further decreased the expression of Cdk2, Cdk4, Cyclin D1 and Cyclin E proteins. In addition, Fig. 5D showed MPE inhibited PI3K/Akt signaling, indicating the effect of MPE on promoting apoptosis and proliferation of cancer cell.

3.4. *MPE caused Hep3B cell death by inducing autophagy pathway*

Autophagy is a multi-step process that involves the formation of the autophagosome, also called acidic vesicular organelles (AVOs), which can be stained with AO [21]. We therefore investigated whether MPE could induce autophagy in HepG2 and Hep3B cells by staining with AO. Fig. 6A showed that MPE promoted the accumulation of AVOs in Hep3B cells but not HepG2 cells. We next validated the target pathway by which MPE signals autophagy in Hep3B cells. The key biological marker to identify autophagy in mammalian systems is the microtubule associated protein 1A/1B-light chain 3 (LC3 II) [22]. In Fig. 6B, MPE induced the formation of LC3 II in Hep3B cells. Upstream regulation of LC3 II occurs via different signaling pathways, including PI3K, Akt, mTOR and AMPK. Down-regulation of PI3K/Akt leads to inactivated mTOR and induce autophagy in cancer cells [23]. AMPK is activated by depletion of ATP, ER stress and oxidative stress, inhibits mTOR, and activates autophagy [24]. Our result showed that treatment with MPE did not affect the expression of PI3K and both decrease in the Akt and p-Akt were observed (Fig. 6B). We further investigated the effect of MPE treatment on mTOR activity. Exposure of Hep3B cells to MPE resulted in



HepG2-PI stain	subG1	G0/G1	S	G2/M
control	3.09	51.85	27.43	18.04
EtOH	5.61	51.04	30.55	13.96
0.25mg/ml	4.32	67.21	18.55	10.59
0.5mg/ml	6.76	79.81	4.19	9.57
1mg/ml	25.51	23.54	39.46	12.12

Hep3B-PI stain	subG1	G0/G1	S	G2/M
control	1.7	59.72	22.93	15.35
EtOH	2.00	58.15	23.47	15.62
0.25mg/ml	1.18	53.98	27.88	17.35
0.5mg/ml	1.33	57.87	24.72	16.10
1mg/ml	2.83	63.80	20.95	10.23

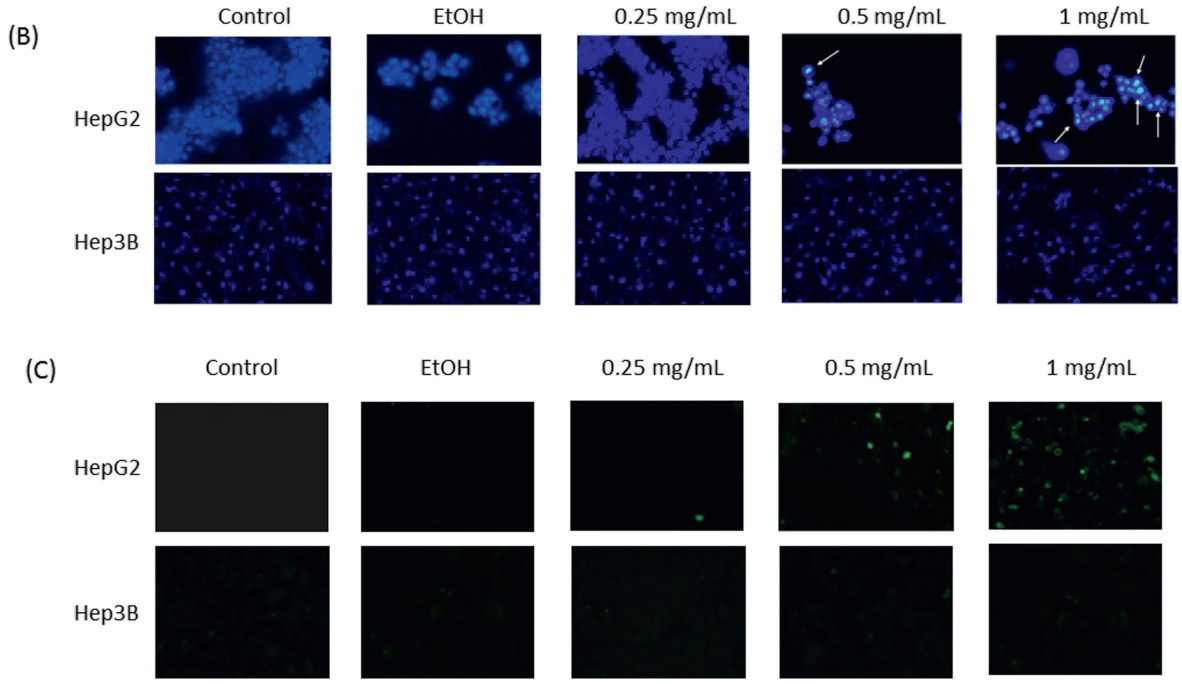


Fig. 4 – MPE induced the morphology changes related to apoptosis in HepG2 cells. HepG2 and Hep3B cells were treated with or without MPE for 24 h. (A) Cell cycle was analyzed by flow cytometry. The apoptosis cells were assayed by DAPI (B) and TUNEL (C) stain. The arrows indicated chromatin condensation and apoptotic cells. The results were shown as the means ± SD of three independent experiments.

diminished levels of phosphorylated form of mTOR. MPE treatment also induced an increase in the phosphorylation of AMPK, revealing a potent effect of MPE on inhibiting PI3K/AKT/mTOR signaling and activating AMPK phosphorylation leading to cell autophagy in HCC cells treated with MPE.

4. Discussion

Increasing evidence revealed that a diet rich in natural polyphenols could lower the risk of liver cancer [7,25]. Polyphenols strictly performs in the role of antioxidant properties which have been proposed as multiple effects involved in anti-carcinogenesis to liver cancer cells, such as apoptosis, anti-proliferation, anti-angiogenesis, inhibition of invasion, and metastasis. Berries are rich in nutritive compounds, including minerals, vitamins, dietary fibers, and in non-nutritive elements, especially polyphenolic phytochemicals [26]. Blackberry (*Rubus* sp.), blackcurrant (*Ribes nigrum*), blueberry (*Vaccinium corymbosum*), raspberry (*Rubus idaeus*), and strawberry (*Fragaria x ananassa*) are most common berries which are usually consumed in fresh and processed forms in the human diet all around the world. Especially, mulberry is an important medicinal plant belonging in Taiwan, and several phenolic

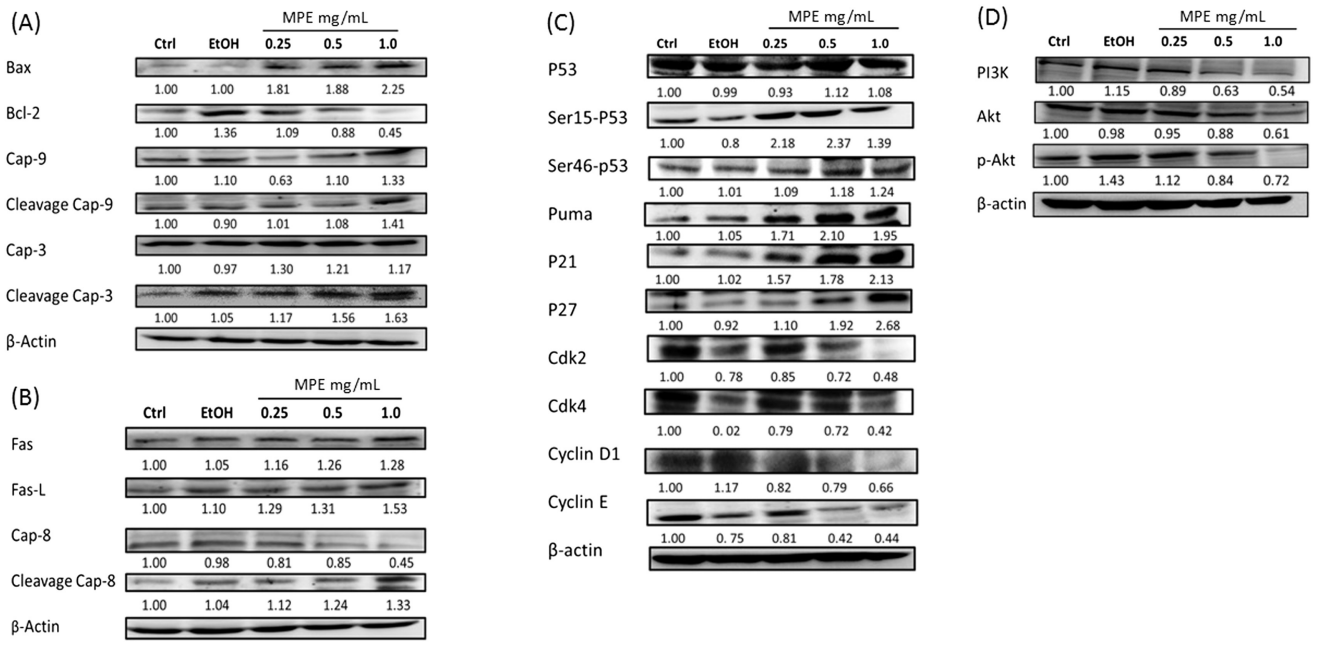


Fig. 5 – MPE modulated P53 phosphorylation and triggered apoptosis and PI3K/Akt pathways to induce HepG2 cell death. HepG2 cells were treated with or without MPE for 24 h. Total cell lysates were prepared and subjected to SDS-PAGE followed by Western blot. The membranes were probed with apoptotic intrinsic (A, extrinsic (B), cell cycle (C) and PI3K (D) related antibodies. The results were represented by using an ECL system. β-actin was the loading control.

compounds and many flavonoids have been isolated from this plant [27]. In this study, we investigated the protective effects of Mulberry fruit in DEN-induced HCC in vivo. Further *in vitro* assays demonstrated that MPE caused liver cancer cell death via p53 dependent apoptosis and p53 null autophagy.

Tumor suppressor p53 maintains the integrity of the genome by initiating cell cycle arrest, apoptosis, and senescence in response to cellular stress. 50% human tumors carry mutant p53, and p53 promotes oncogenic functions such as increased proliferation, survival, and metastasis [28]. In liver cells, p53 regulates the mitotic fidelity and DNA ploidy conservation in hepatocytes of both the normal and regenerative liver [29]. If the p53-dependent senescence program in hepatic stellate cells is abolished, the chronic liver damage increases liver fibrosis and cirrhosis. In addition, loss of p53 promotes the transformation of contiguous epithelial cells into HCC [30]. Several researches demonstrate that mutation or deletion of p53 inhibits the apoptosis of damage cells, then affects the development of HCC from initiation to metastasis [31,32]. Both intrinsic and extrinsic apoptotic pathways converge to the caspases pathway that act as death effector molecules in various forms of cell death. Results of this study demonstrated that the MPE triggered two ways of apoptosis by activating caspase-3, -9, and -8 cleavage in HepG2 cells. In addition, caspases are a number of genes involved in mediating apoptosis. Pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins are mainly involved in the regulation of intrinsic pathway of apoptosis. Pro-apoptotic Bax is involved in the regulation of apoptosis via activation of initiator caspases. The *p53* gene is a tumor suppressor gene and it initiates apoptosis by activating expression Bax [33]. In the present study, upregulation of pro-apoptotic Bax and p53 and downregulation of Bcl-2 were observed in MPE-treated HepG2 cells at 24 h after incubation, indicating activation of apoptosis via a p53 dependent mechanism.

On the other hand, the role of p53 independent regulation in HCC still cannot be ignored, even though the mutations in the p53 have been reported in 50% of HCC patients in Asia and South Africa [34]. Our findings indicated that Mulberry polyphenol extracts triggered an autophagic response, which was accompanied by an increase in the level of LC3 II and p-AMPK proteins in p53-null Hep3B cells. Autophagy is a cellular homeostatic mechanism with stress resistance, immunity, antiaging, and pro-tumor or anti-tumor effects [35]. At basal levels, it is critical for maintaining cytoplasmic as well as genomic integrity via lysosome-dependent catabolic process which degrades cell's components in order to recycle substrates to exert optimally and adapt to tough circumstances [36]. However, dysfunction of autophagy contributes to the pathologies of many human diseases, including HCC [37]. Autophagy involves in both the promotion and prevention of cancer, and its dual roles may be changed during tumor progression [38]. It has been proved that autophagy has a suppressive role in the early stage of HCC tumorigenesis. Once HCC is established, autophagy promotes the tumor progression, metastasis, and therapeutic resistance. Thus, autophagy inhibition could effectively suppress HCC growth and metastasis. However, in target HCC therapy, the role of autophagy is uncertain, for either inhibition or promotion, according to the progression of HCC. Notable, some polyphenols can induce tumor cell death through autophagy activation; for instance, *Solanum nigrum* L. extract decreased p-AKT levels causing

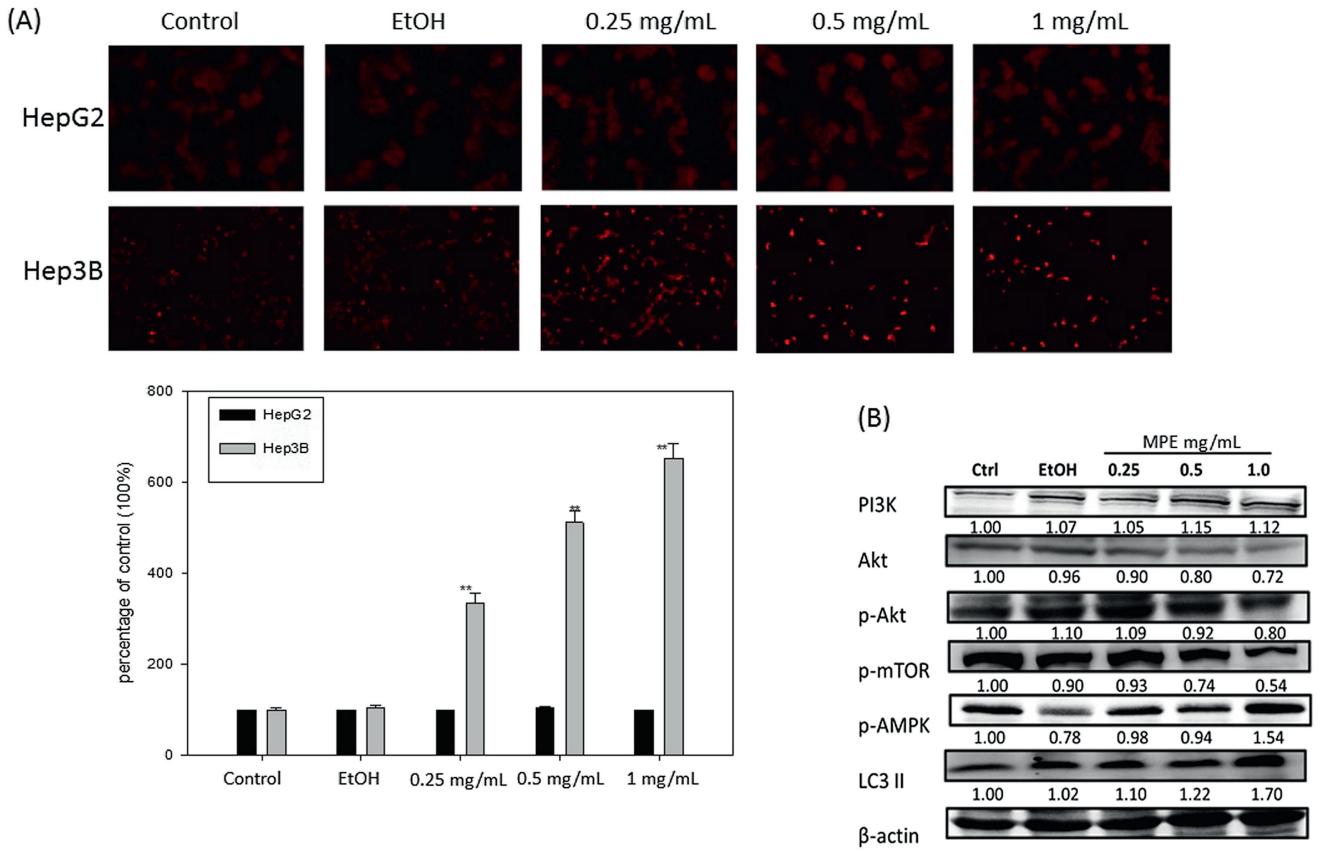


Fig. 6 – MPE caused Hep3B cell death by inducing autophagy pathway. (A) HepG2 and Hep3B cells were incubated with or without MPE for 24 h. The acidic vesicular organelles were assayed by acridine orange and then observed under a fluorescence microscope. Acridine orange uptake was calculated by subtracting mean fluorescent intensity of untreated control cells (autofluorescence). Results are representative of at least 3 independent experiments. (B) Hep3B cell lysates were harvested, and representative blots of PI3K, Akt, p-Akt, p-mTOR, p-AMPK and LC3-II determined by western blotting. β-Actin was used as the internal control.

mTOR inactivation and triggering autophagy in AU565 human breast cancer cells [39], as well as blueberry polyphenols in MDA-MB231 cells through PI3K signal [40], and Litchi fruits extracts in human colon cancer cells [41]. Comprehensive, polyphenols have demonstrated antitumor activity reaching different molecular targets to switch on autophagy in early stage of cancer.

Except apoptosis and autophagy, cells can die by programmed necrosis. TNFα is one of the cytokines to promote inflammation and trigger cell death signaling [42]. In this study, MPE caused about 70% HepG2 cells death (Fig. 3A), however the sub-G1 ratio was 25.51% in 1 mg/mL MPE-treated cells (Fig. 4A). In fact, MPE could prevent hepatocarcinogenesis via regulating inflammatory factors (data not shown), and related research will be published in the future. Beside, our in vivo study revealed that MWE showed the hepatoprotective effect in DEN-treated rats (Table 1). There is no significant difference on two dosages (1%, 2%) of MWE treatment in serum biochemical tests and tumor foci formation, indicating 1% MWE plays a good role in hepatoprotective effect and excessive consumption is useless. In conclusion, mulberry extracts has a potential to be a health supplement to prevent hepatocarcinogenesis in the future.

Conflicts of interest

The authors declare no conflict of interest.

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