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Anticancer activity and mediation of apoptosis in hepatoma carcinoma cells induced by djulis and its bioactive compounds

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ABSTRACT

Anticancer activity and mediation of apoptosis in hepatoma carcinoma cells induced by ethanolic extracts of djulis (EECF) and its bioactive compounds were investigated. EECF (50–500 μg/ml) exhibited significant cytotoxicity in HepG2 cells. AnnexinV-FITC/PI and DAPI staining assay showed that EECF induced apoptosis, accompanied with Sub-G0 phase arrest, ROS generation, loss of mitochondrial membrane potential (ΔΨm), increase of Bax/Bcl-2 ratio, caspase-3 activation and PARP cleavage. According to the HPLC-DAD and HPLC-MS/MS analysis, betanin, rutin, kaempferol, and quercetin were present in EECF, and showed antiproliferative action in HepG2 cells. Among the four bioactive compounds, quercetin showed the highest apoptotic effect toward HepG2 cells. *In vivo*, EECF and quercetin significantly reduced tumor growth in nude mice in tumor xenografts of HepG2 cells. Overall, the presence of bioactive compounds in EECF may account for the anticancer activity of EECF, and EECF could act as a potential chemopreventive agent against growth of hepatoma carcinoma cells.

1. Introduction

Cancer is a leading cause of death worldwide, accounting for an estimated 9.6 million deaths in 2018 (WHO, 2019). According to the WHO's record, liver cancer ranked fourth among the causes of cancer death in 2018. Liver cancer is increasing most rapidly, by 2% to 3% annually during 2007 through 2016, although the pace has slowed from previous years in the United States (Siegel & Miller, 2020). Between 30 and 50% of cancers can be prevented by avoiding risk factors (WHO, 2020). Meanwhile, cancer burden can also be reduced through early detection of cancer. In other words, many cancers have a high chance of cure if diagnosed early and treated adequately (WHO, 2019). Cancer can be treated by radiation, surgery, monoclonal antibody therapy, chemotherapy and other medical methods that aim to eliminate cancer recurrence. However, clinical cancer treatments nowadays still face recurrence due to the residual cancer cells and minute lesions in surgery, chemotherapy and other treatments (Li et al., 2018). Thus, there is urgent need to search for and develop novel and new anticancer agents that are safe and effective. Accumulating evidence shows that natural phytochemicals play an important role in the prevention of cancers with few side effects (Sun & Liu, 2008; Xiang et al., 2016). For, example, dark sweet cherry phenolics enriched in phenolic acid, flavonoids and proanthecyanidins inhibited breast cancer cell growth without toxicity to normal breast cells (Lage et al., 2020). Curcumin and isoflavonoids could be used for cancer chemotherapy (Mark, Lyu, Lee, Parra-Saldívar, & Chen, 2019). Therefore, many studies have focused on the protective effects of natural sources against cancer cells.

Research has shown that there are three types of programmed cell death, apoptosis, autophagy and non-lysosomal cell death (Li et al., 2018). Apoptosis is considered as an important component of various processes including normal cell homeostasis, immune system development, and chemical-induced cell death (Zhao et al., 2018). A number of studies have been reported that some chemopreventive agents exert their anticancer activity by regulating apoptosis, thereby leading to potential for new drugs that could be useful for anticancer treatment (Yuan, Wei, Wang, & Liu, 2014).

Apoptosis may be triggered by several upstream signaling pathways. Numerous studies have shown that many anticancer compounds target

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signaling intermediates in apoptosis-inducing pathways (Lin et al., 2012). Thus, exploring signaling pathways involved in induction of apoptosis in cell lines may elucidate the mechanism of apoptosis induced.

Djulis (*Chenopodium formosanum*) is a cereal plant that contains valuable nutrients and bioactive compounds (Chyau, Chu, Chen, & Duh, 2015). Recent studies reported that djulis has antioxidant and antidiabetic potentials and protects the skin against UV-induced damage (Chyau, Chu, Chen, & Duh, 2015; Hong, Huang, Liu, & Tsai, 2016; Hsu, Pan, Wu, Ho, & Hwang, 2018). In addition, djulis has been shown to prevent colon carcinogenesis due to antioxidant and apoptotic effect (Lee, Chen, Xie, & Shih, 2019). Apart from these studies, the previous studies by the authors reported that bioactive compounds such as rutin, betanin, kaemperol, quercetin and other compounds are present in djulis (Chyau, Chu, Chen, & Duh, 2015). Also, djulis and its bioactive compounds have demonstrated hepatoprotection (Chen, Chu, Chyau, Fu, & Duh, 2018; Chu et al., 2016), antiadipogenesis (Chyau, Chu, Chen, & Duh, 2015), inhibition of hyperlipidemia and hyperglycemia (Chen, Chu, Lin, & Duh, 2019), and antihypertension (Chen, Chu, Chyau, Yang, & Duh, 2019). Many studies showed that bioactive compounds such as rutin, quercetin, and keampferol induced apoptosis in human cancer cells (Abotaleb et al., 2019; Caparica, Júlio, Araújo, Baby, Fonte, & Costa, 2020; Rauf et al., 2018). Given that djulis has demonstrated marked biological effects, as mentioned above, it is possible that hepatoma cell growth may be inhibited by djulis. However, there are no reports concerning the anticancer effects of djulis and its bioactive compounds on hepatoma HepG2 cells. Therefore, the present study aims to explore the anticancer effects of djulis and its bioactive compounds on hepatoma cells, *in vitro* and *in vivo*, and to elucidate the potential mechanism.

2. Materials and methods

2.1. Sample preparation

Djulis (*Chenopodium formosanum*) which was dehusked, identified by Professor Yau-Lun Kuo of the Department of Forestry, National Pingtung University of Science and Technology, was purchased from Kullku Farm, Pingtung, Taiwan, and was ground to a fine powder before the extraction. The voucher specimen (No. CNU-101) was deposited in the Department of Food Science and Technology, Chia Nan University of Pharmacy and Science. The powder (10 g) was extracted with 100 ml of 50% (*v/v*) ethanol and stirred for 16 h, at room temperature. The extracts were filtered through Advantec No 2 filter paper (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) in duplicate under the same conditions. After being filtered, the concentrated supernatant was then combined and concentrated in a rotary evaporator under vacuum, lyophilized to dryness. The ethanolic extracts of djulis, abbreviated as EECF, was stored at −20 °C until used (Chyau, Chu, Chen, & Duh, 2018).

2.2. Cell culture and cell viability

HepG2 cells (ATCC number: CRL-11997), a human hepatoma cell line, were purchased from Bioresources Collection and Research Center (Shin-Chu, Taiwan). HepG2 cells were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 2 mM glutamine, 100 units/ml of penicillin, and maintained in humidified 5% CO_2/95% air at 37 °C. The tetrazolium dye colorimetric test (MTT test) was used to determine the viability of HepG2 cells. Cells were plated in 96-well flat-bottom plat at a density of 2×10^4 cells/well and grown. After 24 h incubation, cells were treated with or without 50, 250, and 500 μg/ml EECF, and 50, 100, and 200 μM bioactive compound. After 72 h incubation, 50 μl 0.1% MTT solution was added and cells were incubated for a further 1 h. Subsequently, the reaction was terminated and the plates were incubated for 30 min to solubilize the formazan dye by addition of 100 μl dimethyl sulfoxide. The optical density of each well was determined at 550 nm using ELISA reader (Molecular Devices VMax, MA, USA) (Denizot, Wilson, Battye, Berke, & Shortman, 1986).

2.3. Cell leakage rate

HepG2 cells were plated in 24-well flat-bottom plat at a density of 5×10^4 cells/well and grown. After 24 h incubation, washed and replaced with fresh medium, then cells were treated with or without 50, 250, and 500 μg/ml EECF, and 50, 100, and 200 μM bioactive compound. After 72 h incubation, 10 μl of extracellular fluid from cell culture and 100 μl of lactate dehydrogenase (LDH) reagent (LDH-Cytotoxicity Colorimetric Assay Kit II, BioVision, Asia Bioscience, Taiwan) were mixed, and incubated for 30 min in dark at room temperature. At the end of the incubation, the absorbance was read at 450 nm using ELISA reader (Molecular Devices VMax, MA, USA). The cell leakage rate was estimated from the ratio between the LDH activity in the medium and that of the whole cell content (Patil et al., 2009).

2.4. Intracellular reactive oxygen species (ROS)

The intracellular levels of ROS in HepG2 cells were assessed by the probe of DCFH-DA, a ROS detection kit, according to the manufacturer's optimized instructions. Cells were plated in the growth medium at a density of 2×10^5 cells/well in 6 flat bottomed well plates and incubated for 24 h. Then washed and replaced with fresh medium. After preincubation for 24 h with 0, 50, 250, and 500 μg/ml EECF, and 0, 50, 100, and 200 μM bioactive compound, they were treated with 50 μM 2′,7′-dichlorodihydrofluorescein diacetate (Merck, Darmstadt, Germany) in a 5% CO_2 light-protected humidified incubator at 37 °C for 30 min. After washing with PBS, the cells were treated with 100 μl Trypsin-EDTA for 3 min and suspended in PBS after centrifugation. The ROS produced from intracellular stress was measured using the microplate fluorescence reader (FLx-800, BioTek, Taiwan) with excitation and emission wavelengths of 485 and 528 nm, respectively (Chyau, Chu, Chen, & Duh, 2015).

2.5. DAPI staining

Approximately 5×10^4 cells/well were seeded in 24 well cell culture plates. Cells were treated with or without 50, 250, and 500 μg/ml EECF, and 50, 100, and 200 μM bioactive compound at 37 °C for 72 h and washed 2 times with PBS. Then 0.5 ml 4% paraformaldehyde was added to each well and incubated for 30 min at 37 °C. Afterwards, the wells washed with PBS, 0.5 ml of Triton-X-100 solution (0.1%) was added to each well and incubated for 2 min at 37 °C. After that, the wells were rewashed with PBS and 0.5 ml DAPI (40, 60-diamidino-2-phenylindole) solution (1 μg/ml) was added to each well and was incubated for 5 min. Finally, the wells were washed with PBS and the cells with condensed and fragmented chromatin were analyzed with fluorescence microscope (cytation 5, Biotek, USA) at 400 × magnifications. (Chyau, Chu, Chen, & Duh, 2015)

2.6. Cell cycle analysis

Cell cycle was assayed by flow cytometry. The cells were seeded at a density of 1×10^6 cells/ml in 6 cm dish and cultured for 24 h. After being cultured, the cells were treated with 500 μg/ml EECF for 72 h. The cells were washed twice with PBS and fixed in 70% ice-cold ethanol overnight. The sample was concentrated by removing ethanol and treating with 800 μl PBS, adding 100 μl RNase (50 μg/ml), incubating at 37 °C for 2 h, then adding 100 μl propidium iodide and incubating at room temperature for 10 min in dark. The cell cycle distribution was analyzed by flow cytometry (Park et al., 2007).

2.7. Apoptosis assay by flow cytometry

The apoptosis of cells induced by the extracts was assayed by treatment with Annexin V and propidium iodide (PI) double labeling according to the manufacturer's instruction (Annexin V-FITC Apoptosis Detection Kit, Biovision, Asia Bioscience, Taiwan). The cells were seeded at a density of 1×10^6 cells/ml in 6 cm dish and cultured for 24 h. Cells were incubated for 72 h in the presence or absence of 50, 250, and 500 μg/ml EECF. Cells were centrifuged to remove the medium, washed with PBS, and stained with Annexin V and PI in binding buffer. The stained cells were determined using FACScan flow cytometry (Beckman Coulter, CA, USA). Annexin V^+PI^- cells were considered as early apoptosis while Annexin V^+PI^+ cells as late apoptosis. (Chu, Chen, Chyau, & Duh, 2017)

2.8. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential of cells was determined by JC-1 mitochondrial membrane potential assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). HepG2 cells were seeded at a density of 2×10^5 cells/well in 6 well plates and incubated for 24 h. After treatment with 50, 250, and 500 μg/ml EECF, and 50, 100, and 200 μM bioactive compound for 24 h, cells were incubated with JC-1, at 37 °C for 30 min in a humidified 5% CO_2 incubator. Then, cells were collected and washed with 100 μl of PBS. After the cells were centrifuged (9.3 *g*, 5 min), they were transferred to 96 well dark plates and resuspended in 100 μl of PBS. The mitochondrial membrane potential was measured using the microplate fluorescence reader (FLx-800, BioTek, Taiwan) with excitation and emission wavelengths of 530 and 590 nm, respectively (Chyau, Chu, Chen, & Duh, 2015).

2.9. Measurement of caspase-3 activity

Caspase-3 activity was determined using the BioVision CPP32/Caspase-3 colorimetric assay kit (Asia Bioscience, Taiwan). After a 32-hour incubation with 50, 250, and 500 μg/ml EECF, and 50, 100, and 200 μM bioactive compound, untreated and treated cells (2×10^5 cells/well) were washed in cold PBS, the cells were treated with 100 μl trypsin-EDTA for 3 min and suspended in PBS after centrifugation. Cell lysates were pelleted, followed by transfer of the supernatants to microcentrifuge tubes. Fifty microliters of reaction buffer with 5 mmol/l DTT and 5 μl of 1 mmol/l DEVD-*p*-nitroanilide (*p*NA)-conjugated CPP32 substrate were added to each tube, followed by 1 h incubation in a water bath at 37 °C. Optical density (OD) for each specimen was determined at 405 nm using the ELISA reader (Molecular Devices VMax, MA, USA) (Chyau, Chu, Chen, & Duh, 2015).

2.10. Western blot

The cells were seeded at a density of 1×10^6 cells/ml in 6 cm dish and cultured for 24 h. The cells were treated with 50, 250, and 500 μg/ml EECF, and 50, 100, and 200 μM bioactive compound for 48 h to determine Bax/Bcl-2 and PARP. Cells were collected and lysed in the ice-cold lysis buffer (Cell Lysis Buffer, Cell Signaling Technology, MA, USA). Protein concentration was determined using Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Each sample, which contained 100 μg proteins, was separated on 10% SDS-PAGE on Mini-PROTEAN® electrophoresis system (Bio-Rad Laboratories Inc., Taipei, Taiwan) and transferred into a NC membrane (Sartonets Taiwan Ltd., Taipei, Taiwan). Nonspecific binding site of the membrane was blocked with 5% BSA in PBST (0.1% *v/v* Tween-20 in PBS, pH 7.2) for 1 hr. Then, immunoblotted with specificity primary rabbit antibody (1:1000) against Bcl-2 (#3498, Cell Signaling Technology, MA, USA), Bax (#2772, Cell Signaling Technology, MA, USA), PARP (#9542, Cell Signaling Technology, MA, USA) and β-action (#4970, Cell Signaling Technology, MA, USA) at 4 °C for overnight. After washing, the membrane was incubated with horseradish peroxidase conjugated secondary antibody (1:5000, rabbit anti-rabbit IgG-HRP, Jackson, West Grove, PA, USA). After washing and visualized using an enhanced chemiluminescence kit (GE Healthcare, Chicago, Illinois, USA), and then exposed to Gel Electrophoresis Documentation-Multi-function Gel Image system (Topbio Co., Taiwan, ROC). The relative expression of proteins was quantified densitometrically using the Image J software and calculated according to the reference bands of β-actin (Chu, Chen, Chyau, & Duh, 2017).

2.11. HPLC/ESI-MS analysis of djulis

The HPLC/electrospray ionization (ESI) mass spectrometric analysis was performed as our previous report (Chu et al., 2016). In brief, an Agilent 1260 Infinity HPLC system in connected with 6420 mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) in positive and negative ionization modes was conducted in the study with minor modification. The ethanol-aqueous extracts (10 mg/ml) of djulis were filtered through 0.22 μm membrane filter before being injected into the analysis column (HSST3, 2.1 × 100 mm, 1.8 μm particle size, Waters Corporation, Milford, MA, USA), which was connected with a guard column (Security Guard C18 (ODS) 4 mm × 3.0 mm ID, Phenomenex Inc., Torrance, CA, USA) and was installed in a column oven set at 35 °C. The mobile phase consisted of two solvents: Solvent A (water containing 0.1% formic acid) and Solvent B (acetonitrile containing 0.1% formic acid). The flow rate during the elution process was set at 0.3 ml/min. A linear gradient elution was carried out with 2–5% B in 3 min, 3–15 min, 5–30% B, 15–20 min, 30–95% B, and finally 95% B isocratic elution for 10 min. The absorption spectra of eluted compounds were scanned within 210 to 650 nm using the in-line PDA detector monitored at 280, 360 and 530 nm, respectively. The compounds having been eluted and separated were further identified with triple quadruple (QQQ) mass spectrometer in the operating parameters as follows: nitrogen used both as a drying gas at a flow rate of 9 L/min and as a nebulising gas at a pressure of 40 *psi*, drying gas temperature 325 °C, and a potential of 3500 V applied across the capillary, fragmentor voltage 125 V, and cell acceleration voltage, 7 V. Quadrupole 1 filtered the calculated *m/z* of each compound of interest, while quadrupole 2 scanned for ions produced by nitrogen collision of these ionized compounds in the range 100–800 *m/z* at a scan time of 200 *ms* per cycle and the collision energy 15–30 V. The identification and quantification conditions of separated compounds were the same as previous report (Chu et al., 2016).

2.12. Experimental animals and grouping

Among the bioactive compounds tested, quercetin showed the highest apoptotic effect toward HepG2 cells. Therefore, quercetin was selected for *in vivo* test, along with EECF. BALB/c-*nu/nu*, male nude mice were purchased from BioLASCO Co. (Taipei, Taiwan). HepG2 cells were injected in 6-week-old male nude mice (16–18 g), which were kept in filter top cages in a room maintained at 25 ± 1 °C with 60% humidity. Sterilized food and water were accessible *ad libitum*. The 40 experimental mice were equally randomized into eight groups (five for each group): control (C), negative control (NC), low (5 mg quercetin/kg bw, LQ), medium (20 mg quercetin/kg bw, MQ), high (40 mg quercetin/kg bw, HQ), low (25 mg EECF/kg bw, LCF), medium (50 mg EECF/kg bw, MCF), and high (100 mg EECF/kg bw, HCF) dose groups. The C group was normal mice without implanted hepatoma cells and orally gavaged the equivalent volume of normal saline. The NC group was mice with implanted hepatoma cells and gavaged normal saline in the same way. Three positive control groups (LQ, MQ and HQ) and three groups of experimental mice (LCF, MCF and HCF) were given the indicated dose of quercetin or EECF orally three times a week for 21 days, respectively, starting on the first day after hepatoma cell implantation. All experimental procedures involving animal studies were

conducted in accordance with the National Institutes of Health (NIH). This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) of Chi Mei Medical Center, Tainan, Taiwan. (Chu, Chen, Chyau, & Duh, 2017)

2.13. Subcutaneous injection of tumor cells and measurement of antitumor effects of EECF

The protocol of the mice hepatocarcinogenesis in this study was performed according to Weng et al. method (Weng et al., 2009). Subconfluent cultures of HepG2 cells were detached by trypsinization. The cells were washed three times with serum-free DMEM. Cells were suspended at a concentration of 1×10^8 cells/ml in serum-free DMEM with the equivalent volume of metrigel and then stored on ice for injection. Each nude mouse was implanted with 200 μl (1×10^7 cells/mice) by subcutaneous injection (with a 24-gauge needle) into the hind neck with a 1 ml syringe. Tumor volume was measured once every other day using a caliper when the first tumors formed. Tumor volume was estimated according to the following formula: tumor volume (mm^3) = length width^2/2. All of the mice were sacrificed at day 22 after tumor implantation.

2.14. Statistical analysis

Each experiment was performed at least triplicate and averaged. All data were recorded as means ± SD. Statistical analysis involved use of the Statistical Analysis System software package. Continuous variables were performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests at a level of $p < 0.05$. To assess differences in tumor volume and weight of nude mice with hepatocellular carcinoma xenografts among various groups, a Kruskal-Wallis test was applied, followed by median test at a level of $p < 0.05$.

3. Results

3.1. Effect of EECF on cell viability, cell cycle arrest and apoptosis

Data presented in Fig. 1A show EECF significantly reduced the cell viability in a concentration-dependent manner in different incubation time (24, 48, and 72 h), using the MTT assay. HepG2 cells were more sensitive to EECF, when the cells were treated with EECF for 72 h. The IC_{50} value of EECF in HepG2 cells incubated for 72 h was 349.4 μg/ml. In addition, when the cells were damaged, lactate dehydrogenase (LDH) was released from the cells. Fig. 1B shows a release of LDH in a concentration-dependent manner. These results indicate that EECF can potently inhibit proliferation of HepG2 cells.

The results of cell cycle analysis of cells treated with EECF are shown in Fig. 1C, using flow cytometry. When the cells were treated with EECF 500 μg/ml, the amount of the cells in the Sub-G0 phase increased from 0.62% to 18.8%, compared with the untreated cells. The rate of cells in G0/G1 phase and G2/M phase was reduced significantly from 78.4% to 62.0% and 12.3% to 9.78%, compared with the control. This suggests that a portion of cells were arrested in the Sub-G0 phase after treatment with EECF. In other words, the induction of apoptosis by EECF is cell-cycle-dependent.

In addition, the cells treated with 50, 250, and 500 μg/ml for 72 h, the percentages of early apoptosis plus late apoptosis/necrosis were 7.3%, 9.6% and 55.8% respectively, compared with 3.9% for untreated cells (Fig. 1D). These results confirm the observation of EECF-induced apoptosis. After treating the cells with EECF at different concentrations for 72 h, DAPI staining assay was used to observe whether EECF induced apoptosis in HepG2 cells, followed by Annexin V-FITC/PI double staining assay to measure the percentages of apoptotic cells. Fig. 1E shows that the EECF-treated HepG2 cells displayed different nuclear morphologies compared to the untreated cells, as observed by chromatin condensation and nuclear fragmentation, implying the occurrence of apoptotic characteristics.

3.2. Effect of EECF on HepG2 cells apoptosis

To determine the effect of EECF on mitochondria, the mitochondrial membrane potential (ΔΨm) was determined. As shown in Fig. 2A, when the cells were treated with EECF for 24 h, the ΔΨm value of HepG2 cells significantly decreased in a concentration-dependent manner, compared to the control, indicating EECF induces mitochondria damage. The cells treated with EECF for 48 h showed a significant increase in the ratio of Bax/Bcl-2 in a concentration-dependent manner (Fig. 2B), indicating EECF induces apoptosis in HepG2 cells via alternation of the Bax/Bcl-2 ratio. Treatment of HepG2 cells with different concentrations of EECF resulted in a concentration dependent increase in caspase-3 activity (Fig. 2C). In addition, once caspase-3 is activated, it further hydrolyses the downstream PARP protein. As expected, the data shown in Fig. 2D indicate that the expression level of cleaved PARP increased in the presence of EECF. These results show that EECF may activate caspase-3 activity and then increase the cleavage form of PARP. Oxidative stress is known to be implicated in apoptosis. It is therefore determined that EECF induces ROS generation in EECF. As shown in Fig. 2E, for the cells exposed to EECF for 24 h, a dose-dependent increase in the generation of ROS is observed. EECF at 250 and 500 μg/ml significantly induces ROS generation.

3.3. Analysis of bioactive compounds present in EECF

A previous work by the authors examined the ethanolic extracts of djuis (Chyau, Chu, Chen, & Duh, 2018). However, the contents of bioactive compounds in natural plants may vary due to the differences in extracting solvents, harvesting and storage conditions and climate change (Chyau, Chu, Chen, & Duh, 2018). The extracting solvent was ethanol in this work. It is therefore necessary to determine and identify the bioactive compounds present in EECF in this study. Qualitative and quantitative analyses obtained by HPLC-MS an HPLC-DAD are shown in Table1 and Fig. 3, respectively. Sixteen compounds were identified based on the comparison of their retention times, MS/MS data for standard compounds and published data. Among the identified-compounds, eight were phenolic compounds, including protocatechuic acid derivative, quercetin-3-*O*-rutinoside, 7-*O*-rhamnoside, quercetin-3-*O*-deoxy-hexose-*O*-hexose-*O*-pentoside, kaempferol derivative, rutin, kaempferol-3, 7-di-*O*-rhamnoside, kaempferol-3-*O*-rutinoside and quercetin. Of these phenolic compounds, quercetin, rutin and kaempferol, which are abundant in natural sources, showed significant biological activity (Chen et al., 2018). Aside from these phenolic compounds, betanin, and isobetanin, being responsible for the red color in djulis, are present in EECF. Betanin is a natural pigment with biological activity used in food and pharmaceutical products. Therefore, in the present study, betenin, rutin, kaempferol and quercetin were selected as reference compounds for further evaluating the roles of bioactive compounds in EECF in inducing apoptosis in HepG2 cells.

3.4. Effect of bioactive compounds on apoptosis

The effects of bioactive compounds present in EECF such as betanin, rutin, kaempferol and quercetin on HepG2 cell growth and apoptosis were determined (Fig. 4.). Betanin, rutin, kaempferol and quercetin in the range of 50–200 μM decreased cell proliferation (Fig. 4A) and increased LDH leakage (Fig. 4B). In addition, exposure of HepG2 cells to betanin, rutin, kaempferol and quercetin in the range of 50–200 μM resulted in increasing the levels of apoptotic bodies (Fig. 4C), in a concentration-dependent decrease in ΔΨm (Fig. 4D), and increasing in Bax/Bcl-2 ratio (Fig. 4E), in caspase-3 activity (Fig. 4F) and in cleaved PARP (Fig. 4G) in a concentration-dependent manner compared to the untreated cells. Obviously, betanin, rutin, kaempferol and quercetin

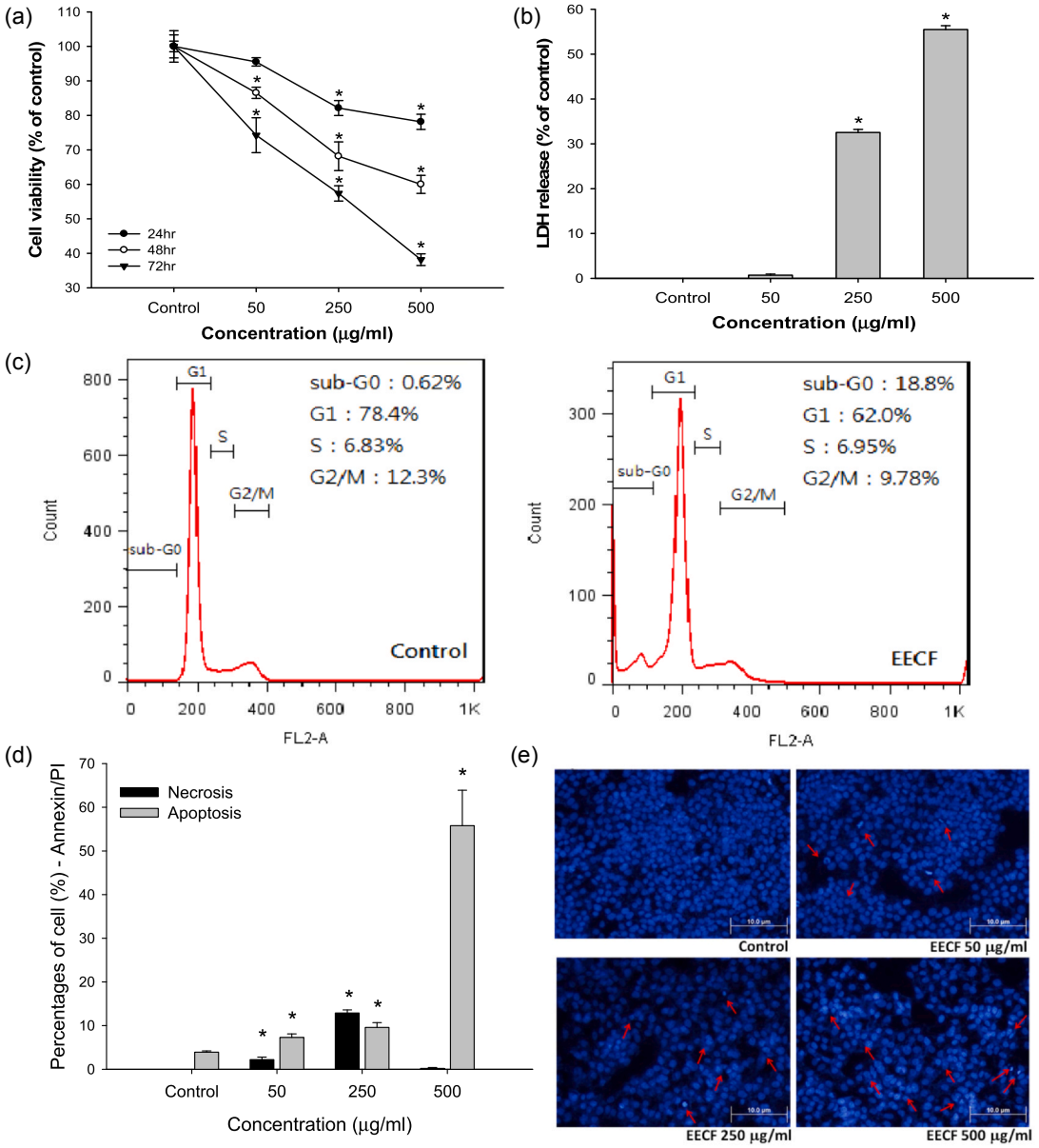

Fig. 1. The cell viability and apoptotic effect of ethanolic extracts of djulis (EECF) on HepG2 cells. (a) Effect of EECF on cell viability. After EECF-treated cells for different incubation time, cell viability was determined by MTT assay. (b) Effect of EECF treatment on cell disruption was determined by lactate dehydrogenase (LDH) leakage assay. The results are expressed as percent of LDH activity in the culture medium relative to the total enzyme activity. (c) Effect of EECF on the cell cycle of HepG2 cells. (d) Apoptotic effect of EECF treatment was assessed by Annexin V-FITC/PI double-staining assay and determined by flow cytometry. (e) Chromatin condensation was determined by DAPI staining after EECF treatment. The DAPI-stained cells were evaluated using fluorescence microscopy (200 x). The arrow indicated apoptotic cells. The cells were incubated with EECF for 24, 48 and 72 h (A), 72 h (B-E), respectively. The data are expressed as the mean ± SD (n = 3). $P < 0.05$ indicated significant difference from the control.

induce apoptosis of HepG2 cells, which may in part account for the antiproliferative effect of EECF. Although betanin, rutin, kaempferol and quercetin demonstrated marked potent antiproliferation of HepG2 cells, quercetin led to the greatest decreases in ΔΨm, caspase-3 and Bax/Bcl-2 ratio. Considering the decrease in ΔΨm, and enhancement of caspase-3 activity and Bax/Bcl-2 ratio, quercetin was selected for *in vivo* test.

3.5. The inhibition of tumor size in a mouse xenograft model

The above results have already shown EECF and its bioactive compounds could act as anticancer compounds *in vitro*. To further verify the anti-tumor effect of EECF and its bioactive compounds, an *in vivo* antitumor study using nude mice with a hepatoma carcinoma HepG2 xenograft model was performed. As shown in Fig. 5A, consumption of EECF at a high (100 mg/kg bw) dose could significantly reduce the growth of HepG2 tumors, compared to the untreated control. Meanwhile, doses of quercetin at 20 and 40 mg/kg bw could significantly inhibit the growth of HepG2 tumors in a mouse xenograft model. The visible tumor sizes are shown in Fig. 5B. Fig. 5C shows the inhibitory effect of EECF and quercetin on the growth of tumor xenografts in nude mice. The data shown in Fig. 5D show the body weights for EECF- and quercetin-treated groups and the untreated control group did not differ significantly.

4. Discussion

Hepatocellular carcinoma (HCC) is one of the most common

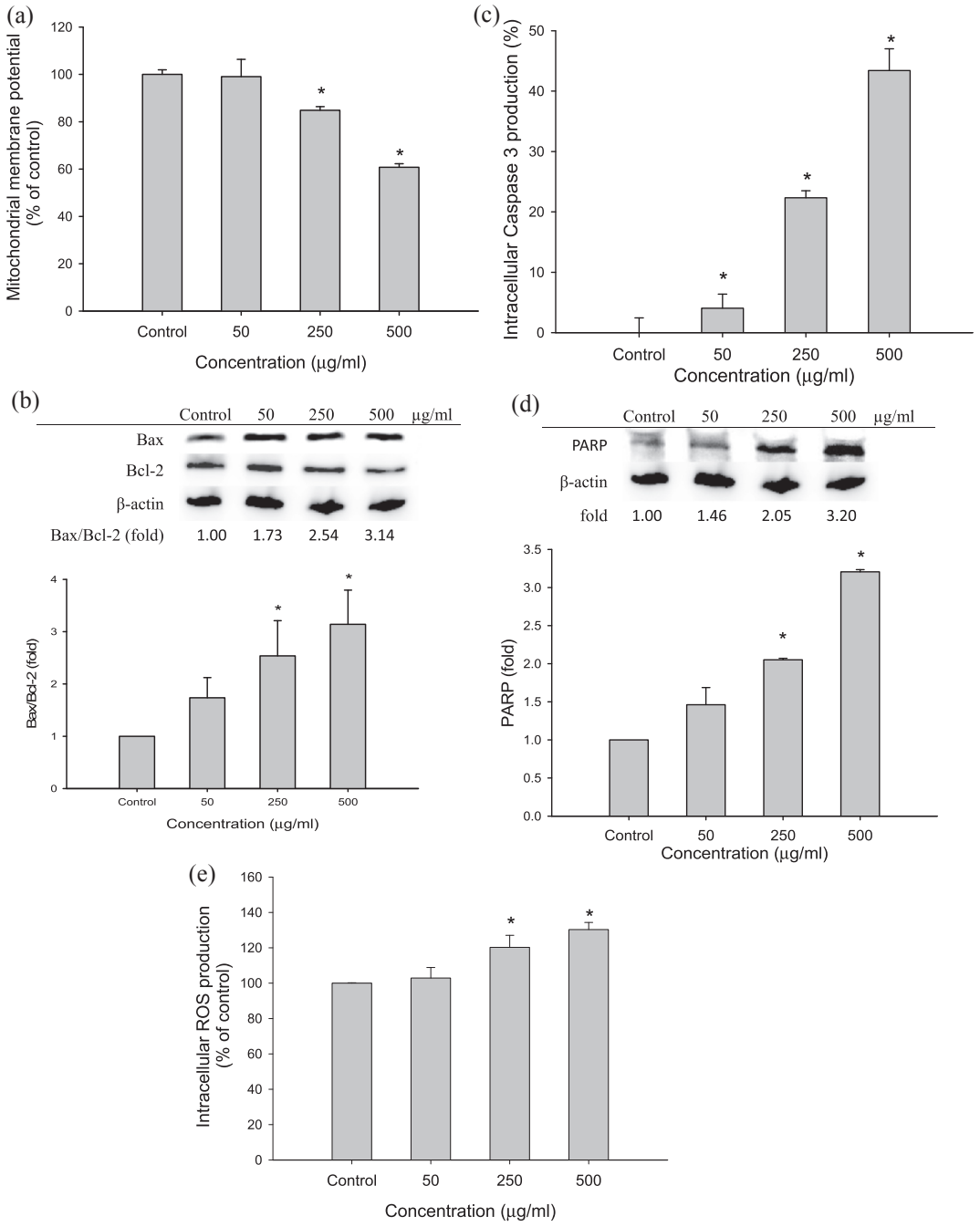

Fig. 2. The effect of different concentrations of ethanolic extracts of djulis (EECF) on HepG2 cells apoptosis. (a) Effect of EECF on mitochondrial membrane potential in HepG2 cells. (b) Effect of EECF on Bax/Bcl-2 ratio in HepG2 cells. (c) Effect of EECF on caspase-3 activity in HepG2 cells. (d) Effect of EECF on PARP cleavage in HepG2 cells. (e) Effect of EECF on reactive oxygen species (ROS). The cells were incubated with EECF for 24 h (a), 48 h (b), 32 h (c) 48 h (d), and 24 h (e), respectively. The data are expressed as the mean ± SD (n = 3). $P < 0.05$ indicated significant difference from the control.

malignant tumors worldwide, accounting for approximately 90% of all primary liver cancers (Zhao, Zhang, Wang, & Chen, 2020). Therefore, there is urgent need to discover new chemopreventive agents that are effective and have no side effects for growth inhibition of hepatocellular carcinoma.

The human hepatocellular carcinoma cell line, HepG2, has been used extensively to study liver cancer (Chandrasekaran, Swaminathan, Chatterjee, & Dey, 2010). Therefore, the current study using HepG2 cells as *in vitro* and *in vivo* models aims to provide insights into the mode of cell death and molecular action mechanism against apoptosis by djulis and its bioactive compounds. A decrease in viability was observed at 72 h when the cells were incubated with EECF, indicating that EECF shows an inhibitory trend on HepG2 cell growth (Fig. 1A). The LDH level in the culture medium is known to be one of markers for predicting cell membrane damage. As expected, EECF increased the release of LDH in HepG2 cells. These observations clearly imply that EECF shows cytotoxic action on HepG2 cell growth, possibly due to disruption of the cell membrane which leads to cell death.

The anticancer activity of EECF was determined further by cell cycle analysis and apoptosis assay by flow cytometry. After treatment with EECF for 72 h, suppression of cell proliferation with EECF at 500 μg/ml was accompanied by significant accumulation of cells in the Sub-G0 phase. These results indicated that treatment with EECF resulted in apoptotic cell death and Sub-G0 phase cell cycle arrest, thereby

Table 1
Retention time, UV–Vis and Mass spectral characteristics of the ethanolic extracts of djulis (*Chenopodiun formosaneum*).

Peak No.	Compound	t_R (min)	λ_{max} (nm)	$[M+H]^+$	Amount (mg/g)[d]
1	protocatechuic acid derivatives	4.67	262, 234	285	33.86
2	unknown	6.61	252,290,532	353$[M+Na]^+$	7.40
3	betanin[a]	6.89	534, 274	551	10.40
4	isobetanin[b]	7.47	534, 236	551	7.75
5	unknown	10.87	315,535	773	6.16
6	quercetin-3-*O*-rutinoside-7-*O*-rhamnoside	11.58	254, 354	757	9.31
7	quercetin-3-*O*-deoxy-hexose-*O*-hexose-*O*-pentoside	12.01	254, 352	743	21.40
8	kaempferol derivative[c]	12.52	268, 348,236sh	697	1.53
9	rutin[a]	12.77	222,256,352	611	26.66
10	unknown	12.85	222,280	245$[M+H]^-$	6.89
11	20-hydroxyecdysone	13.18	246,224sh	481	14.45
12	kaempferol-3,7-di-*O*-rhamnoside[c]	13.38	350,266,234	579	2.08
13	kaempferol-3-*O*-rutinoside[a]	13.77	267,350,235sh	595	3.85
14	unknown	14.27	222,256,348	551	5.48
15	unknown	16.70	222,312,412	659	5.36
16	quercetin	17.38	222,370	303	2.41

[a] The identification was confirmed further by authentic compound.
[b] Compounds were tentatively identified according to mass spectra and the matched data.
[c] Compounds were limitedly identified from mass spectra and UV–visible absorbance spectra.
[d] Peak 4 was quantified as equivalent to betanin and all the others were quantified as quercetin (three replicates for all compounds) based on the amount of mg/g ethanolic extracts of djulis (EECF).

delaying the progression of the cell through the G1, S and G2/M phase in HepG2 cells (Chen et al., 2015).

Expression of proteins involved in apoptosis is taken as a maker regarding the execution of molecular actions (Shen, Chen, & Duh, 2012). Mitochondrion-dependent apoptosis is controlled by Bcl-2 family members (Hsiao et al., 2013). The Bcl-2 family, together with mitochondria, cytochrome *C* and caspase, have been identified as essential components of apoptotic signaling pathways (Chu, Chen, Chyau, & Duh, 2017). The anti-apoptotic members of the Bcl-2 family, such as Bcl-2 or Bcl-xL, inhibit the efflux of these proteins; whereas the pro-apoptotic members, Bax or t-Bid, trigger their release (Lin et al., 2012). Therefore, the ratio of Bax/Bcl-2 is a biochemical marker for the evaluation of tumor/cancer inhibition (Patil et al., 2009). As expected, EECF induces the ratio of Bax/Bcl-2, dose-dependently, indicating that EECF induces apoptosis and is anticarcinogenic. In addition, the loss of MMP, serving as a key step in the mitochondria-dependent apoptosis, provided further proof of the intrinsic apoptotic cascade induced by EECF (Lin et al., 2012). Moreover, in the current study, an increased in the Bax/Bcl-2 protein ratio, loss of mitochondrial membrane potential and activation of caspase-3 and PARP occurred in HepG2 cells after treatment with EECF, suggesting that EECF inducing apoptosis in HepG2 cells might partly occur through a mitochondrion-mediated pathway (Hsiao et al., 2013).

ROS plays a crucial role in cell growth and apoptosis (Chu, Chen, Chyau, & Duh, 2017). This role may be a double-edge sword, because ROS can initiate cell transformation by causing alterations leading to mutations during DNA replication, whereas in already transformed cells, ROS plays an important role in the initiation and execution of apoptosis (Juan, Wenzel, Daniel, & Planas, 2008; Kroemer, Galluzzi, & Brenner, 2007). In addition, excessive ROS generation can also induce signaling pathways, including those involved in oxidative stress, cell cycle arrest and apoptosis (Chu, Chen, Chyau, & Duh, 2017; Moon et al., 2010). The data in Fig. 2E show that EECF may enhance oxidative stress toward HepG2 cells. Many researchers have reported that oxidative stress could cause cellular apoptosis via both the mitochondria-dependent and mitochondria-independent pathways (Sinha, Das, Pal, & Sil,

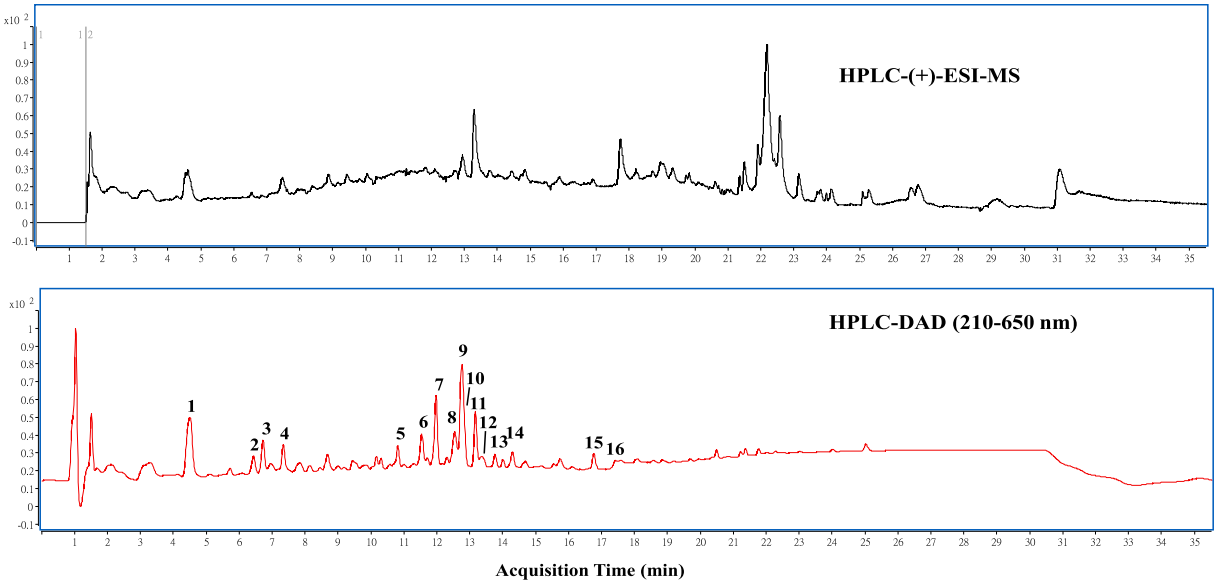

Fig. 3. Total ion chromatograms of LC/MS analysis in positive electrospray ionization and HPLC-photodiode array detection chromatograms at full scan of 210–650 nm from aqueous ethanolic extracts of djulis (EECF). Peak numbers are referred to Table 1.

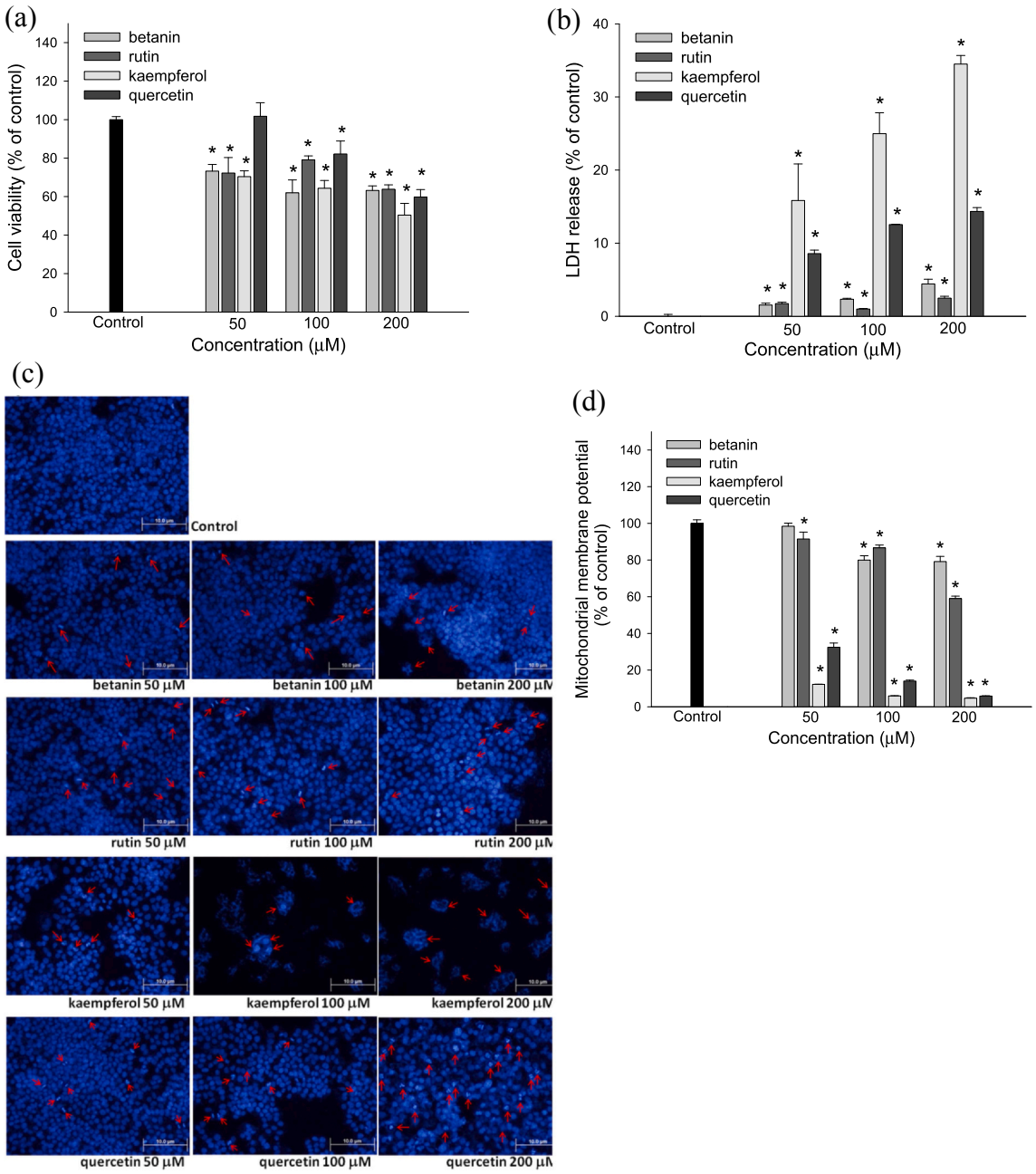

Fig. 4. The effect of betanin, rutin, kaempferol or quercetin on cell viability and apoptotic effect of HepG2 cells. (a) Effect of betanin, rutin, kaempferol or quercetin on cell viability was determined by MTT assay. (b) Effect of betanin, rutin, kaempferol or quercetin on cell disruption was determined by lactate dehydrogenase (LDH) leakage assay. The results are expressed as percent of LDH activity in the culture medium relative to the total enzyme activity. (c) Chromatin condensation was determined by DAPI staining after betanin, rutin, kaempferol or quercetin treatment. The DAPI-stained cells were evaluated using fluorescence microscopy (200 x). The arrow indicated apoptotic cells. (d) Effect of betanin, rutin, kaempferol or quercetin on mitochondrial membrane potential in HepG2 cells. (e) Effect of betanin, rutin, kaempferol or quercetin on Bax/Bcl-2 ratio in HepG2 cells. (f) Effect of betanin, rutin, kaempferol or quercetin on caspase-3 activity in HepG2 cells. (g) Effect of betanin, rutin, kaempferol and quercetin on PARP cleavage in HepG2 cells. The cells were incubated with betanin, rutin, kaempferol or quercetin for 72 h (a), 72 h (b), 72 h (c), 24 h (d), 48 h (e), 32 h (f), and 48 h (g), respectively. The data are expressed as the mean ± SD (n = 3). $P < 0.05$ indicated significant difference from the control.

2013). Excessive ROS production can lead to oxidation of macromolecules and has been implicated in mtDNA mutations, ageing, and cell death (Ott, Gogvadze, Orrenius, & Zhivotovsky, 2007). Interestingly, this significant increase in ROS generation is accompanied by inhibition of cell growth, cell cycle arrest, loss of MMP, increase of the ratio of Bax/Bcl-2, up-regulation of caspase-3 activity and PARP cleavage. Apparently, the induction of oxidative stress by EECF may contribute to apoptosis of HepG2 cells. On the other hand, in our previous study, *t*-BHP-induced ROS generation and lipid peroxidation in HepG2 cells was attenuated by water extracts of djulis (Chyau, Chu, Chen, & Duh, 2015). A reasonable explanation is that the observed cytoprotective potential of water extracts of djulis may reflect mainly their direct actions on mediators of *t*-BHP toxicity, leading to reduction of ROS generation and lipid peroxidation (Lima, Fernandes-Ferreira, & Pereira-Wilson, 2006). It is well known that phenolic compounds play an important role in protection against oxidative damage. However, the initial electron transfer oxidation of phenolics present in natural sources with O_2 generates semiquinone and superoxide anion, subsequently,

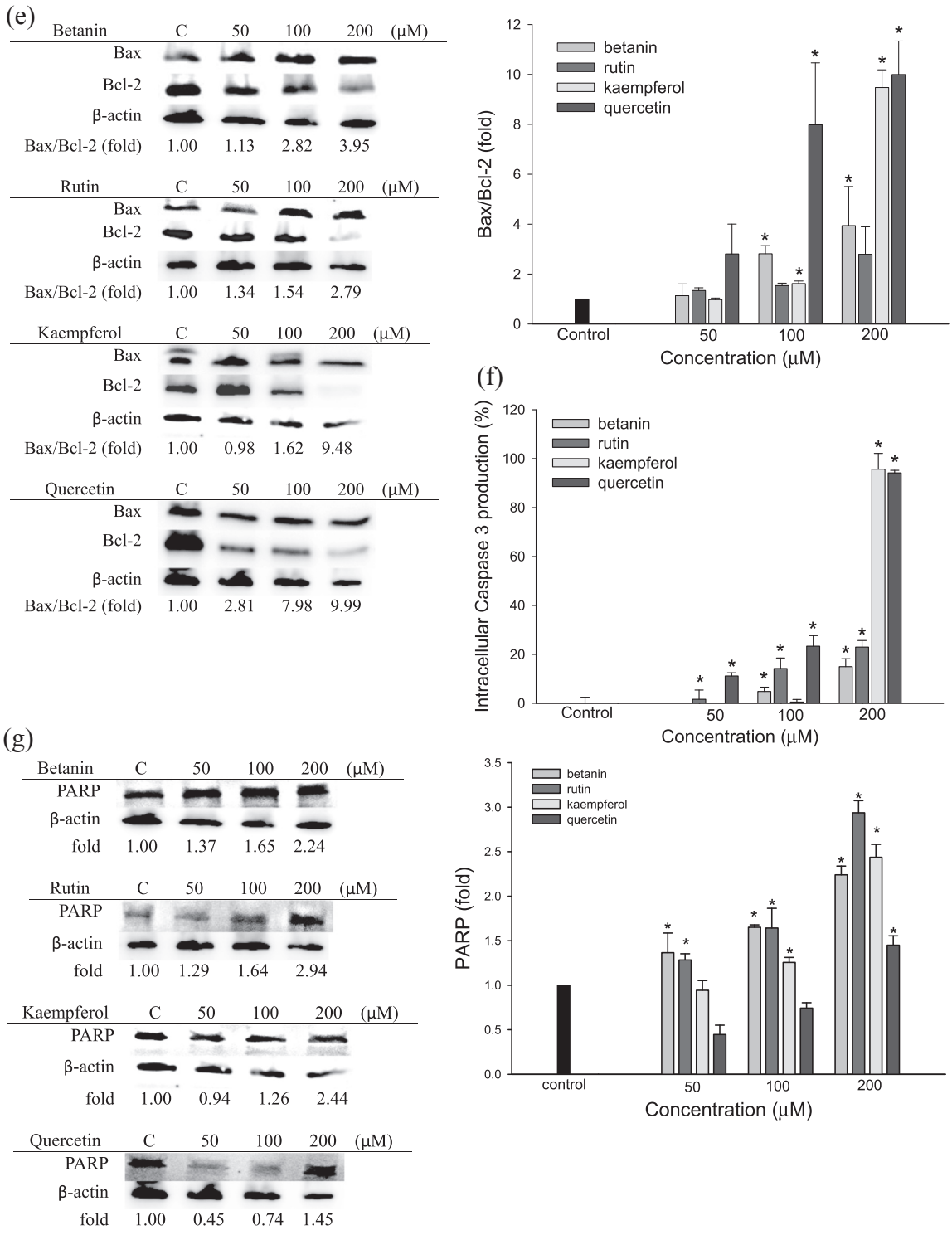

Fig. 4. (*continued*)

superoxide anion reacts with Cu(I), given hydrogen peroxide which reacts with metal ions using Fenton reaction to form hydroxyl radicals to accumulate ROS, thereby inducing oxidative stress and ultimately causing cell death (Lee, Chen, Yu, Wang, & Duh, 2012; Qian et al., 2009). In addition, naringenin and naphthoquinone have been shown to increase ROS generation might result from the decrease GSH/GSSG and the concentration of enzymatic antioxidants (Abotaleb et al., 2019; Sun et al., 2016). Although the influence of EECF on the mechanism action of ROS generation was not conducted in this study, some bioactive compounds such as tea polyphenolics, resveratrol and pinostrobin inducing the death of cancer cells via the prooxidant property may inhibit carcinogenesis (Cianciosi et al., 2020; Mao, Gu, Chen, Yu, & He, 2017). Therefore, we suggest that EECF inhibits HepG2 cells partly due to the generation of ROS, which is induced by EECF. However, further experiments are necessary to elucidate the mechanism action of ROS generation induced by EECF.

Many studies have noted that naturally occurring compounds, known as phytochemicals, are thought to be highly responsible for the protective health benefits against human diseases. Given that sixteen compounds have been identified and are present in EECF, they might

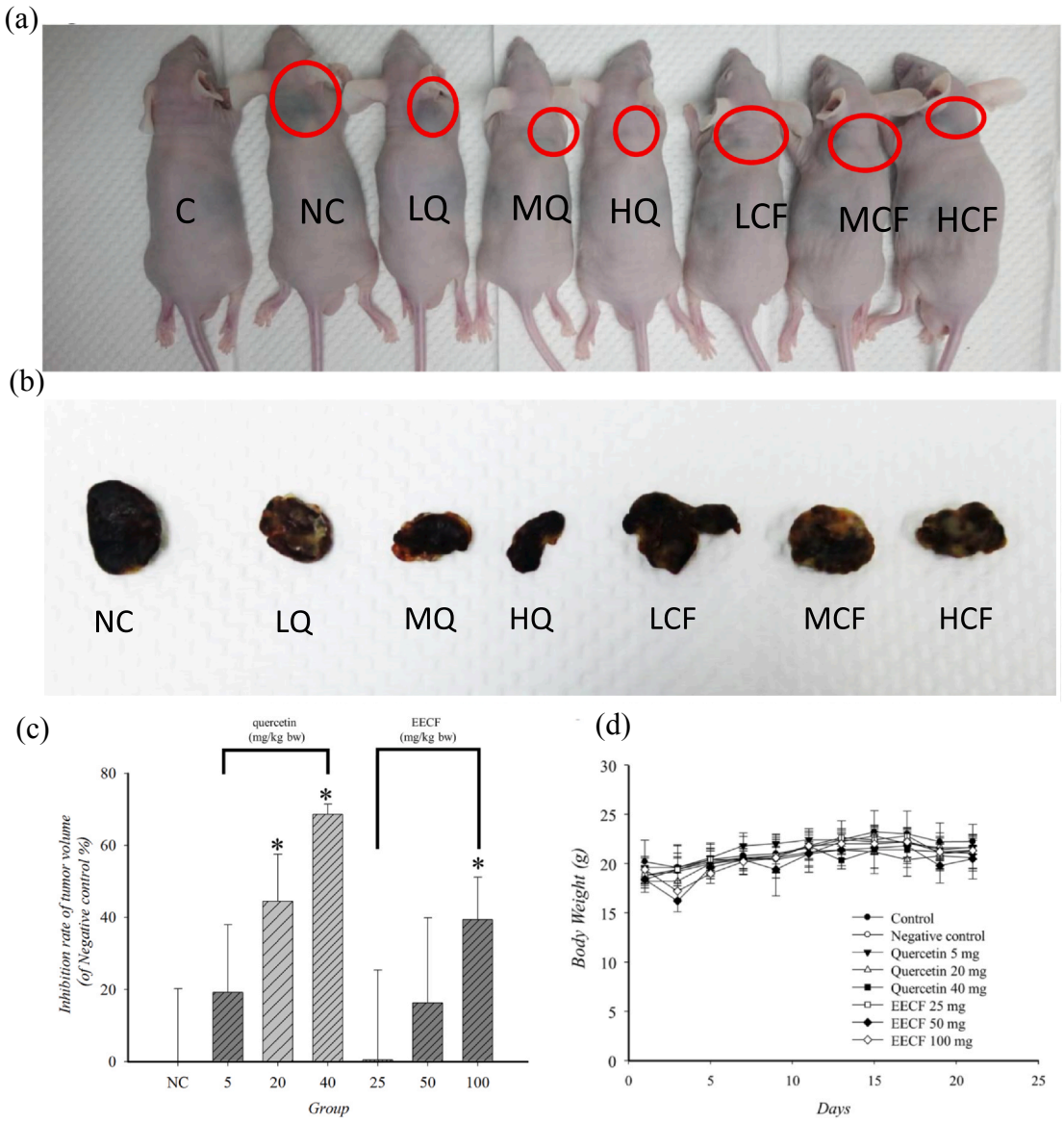

Fig. 5. Effect of ethanolic extracts of djulis (EECF) and quercetin on human tumor growth in xenograft model. (a) Tumor size of representative animals on day 21. C: control; NC: negative control; LQ: quercetin (10 mg/kg bw); MQ: quercetin (20 mg/kg bw); HQ: quercetin (40 mg/kg bw); LCF: EECF (25 mg/kg bw); MCF: EECF (50 mg/kg bw); HCF: EECF (100 mg/kg bw). (b) The animals were sacrificed after 21 days, and solid tumors were collected. NC: negative control; LQ: quercetin (10 mg/kg bw); MQ: quercetin (20 mg/kg bw); HQ: quercetin (40 mg/kg bw); LCF: EECF (25 mg/kg bw); MCF: EECF (50 mg/kg bw); HCF: EECF (100 mg/kg bw). (c) Inhibitory effects of EECF and quercetin on tumor growth. (d) Body weight of animals. The different concentrations of EECF and quercetin were administered orally for 21 consecutive days, starting the day after implantation of tumor cells. Values represent the mean ± SEM (n = 5). Results were statistically analyzed with Dunnett's test. $^{\#}(p < 0.05)$, compared to the control, and * $(p < 0.05)$, compared to the negative control.

play a crucial role in the prevention or attenuation of proliferation of cancer cells associated with inducing apoptosis. It is necessary to determine whether bioactive compounds such as betanin, rutin, kaempferol and quercetin are responsible for inducing the apoptosis of HepG2 cells. Therefore the effects of bioactive compounds on the growth of HepG2 cells, LDH leakage, apoptotic body, change in ΔΨm and Bax/Bcl-2 ratio, and activation of caspase-3 and PARP in HepG2 cells were further investigated. As expected, the decrease in HepG2 cell growth (Fig. 4A) and increase in LDH leakage (Fig. 4B) parallel the cytotoxic effect of betanin-, rutin-, kaempferol- and quercetin-induced cells, indicating betanin, rutin, kaempferol and quercetin induced cytotoxicity via disruption cell membranes (Chu, Chen, Chyau, & Duh, 2017). These findings suggest that EECF has an antiproliferative effect toward HepG2 cells, partly due to betanin, rutin, kaempferol and quercetin present in EECF. Moreover, the results confirmed that betanin, rutin, kaempferol and quercetin significantly increased the apoptotic bodies, reduced ΔΨm, and enhance the ratio of Bax/Bcl-2, activation of caspase-3 and levels of cleaved PARP. In addition, of the four bioactive compounds, betanin at concentrations of 50–200 μM significantly increases ROS generation in HepG2 cells (data not shown). The increase in ROS generation by betanin parallels the decrease in HepG2 cell growth. Therefore, the induction of ROS generation by betanin may partly contribute to antiproliferation of HepG2 cells. Meanwhile, after the cells were treated with rutin, kaempferol or quercetin at the concentrations of 50–200 μM, the ROS generation was reduced (data not shown), confirming the inhibition of ROS generation by rutin, kaempferol or quercetin. This finding is in agreement with that reported by Azevedo et al. (2013), in that rutin, quercetin and kaempferol are polyphenolic compounds with antioxidant properties (Azevedo et al., 2013; Chen & Chen, 2013). The biological activities of flavonols such as

rutin, quercetin and kaempferol are well documented (Chen et al., 2013). These bioactive compounds invoke several different mechanisms in the regulation of cancer cells. Not only are they potent promoter of apoptosis, but they also modify a host of cellular signaling pathways (Chen et al., 2013; Ramos, 2007). In the current study, rutin, kaempferol and quercetin show no effects on inducing oxidative stress (data not shown), however, rutin, kaempferol and quercetin inhibit HepG2 cell growth through apoptosis by activating intrinsic pathways (Fig. 4). As for betanin, inhibited- HepG2 cell growth results from apoptosis and oxidative stress derived from the excessive amount of ROS. Taken together, these observations indicate that betanin, rutin, kaempferol and quercetin may be considered anticarcinogenic, and thereby may contribute to the anticancer activity of EECF.

Accumulating studies show that many biological compounds exhibit anticancer activity. For instance, the antitumor activity of rutin, kaempherol and quercetin might be achieved by immunomodulatory properties (Liu et al., 2012). Betanin, for example, plays an important role in chemoprevention against lung and skin cancers (Chhikara, Kushwaha, Sharma, Gat, & Panghl, 2019). In addition, according to the data in Table 1 and Fig. 3, there are twelve compounds, along with betanin, rutin, kaempferol and quercetin, and other unidentified peaks in EECF, which might render effect, and contribute to the anticancer activity by a direct or a synergistic action through combination of bioactive compounds present in EECF (Chu, Chen, Chyau, & Duh, 2017). That is to say, the bioactive compounds present in EECF may account for its antiproliferative effect on HepG2 cells.

To assess the antiproliferative effect of EECF and its bioactive compound, quercetin *in vivo*, a HepG2 cell xenograft model was used in nude mice. According to the data in Fig. 5C, EECF and quercetin significantly suppressed tumor cell growth. Apparently, EECF and quercetin showed a significant effect and selectively triggered cancer cell death by inducing the classical apoptotic pathway, *in vitro*, and suppressing the proliferation of human hepatoma cells, *in vivo*. These results point to the chemopreventive roles of EECF and quercetin in the blockading of tumor progression in liver cancer. This indicates EECF and quercetin significantly suppress liver tumor growth *in vivo*. Therefore, EECF is safe and available for use in preclinical testing of hepatocellular carcinoma (HCC). However, their relevance needs to be verified.

5. Conclusions

In summary, the present study first demonstrated that EECF has an antiproliferative effect on HepG2 *in vitro*. Most significantly, EECF inhibited the growth of liver tumor xenografts in nude mice. This chemopreventive effect of EECF can be attributed to its production of the up-regulation of LDH leakage, causing cell cycle arrest at the Sub-G0 phase, inducing mitochondria membrane potential loss and enhancing ROS generation, Bax/Bcl-2 ratio, activating caspase-3 activity and PARP cleavage. In addition, the presence of bioactive compounds in EECF may in part be responsible for the antiproliferative action of EECF. Therefore, it is plausible that djulis consumption could inhibit HepG2 cell proliferation accompanied with cell apoptosis in tumor tissue. These results provide a new insight into the chemopreventive characteristics of EECF, which could be developed as anti-hepatoma cells. These findings lead to importance in healthy food and agricultural applications, adding to the value of this plant.

CRediT authorship contribution statement

Chin-Chen Chu: Conceptualization, Resources, Methodology, Funding acquisition. **Shih-Ying Chen:** Conceptualization, Validation, Methodology, Formal analysis. **Charng-Cherng Chyau:** Investigation. **You-Chia Wu:** Investigation. **Heuy-Ling Chu:** Investigation. **Pin-Der Duh:** Conceptualization, Resources, Methodology, Investigation, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.104225.

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