Inhibitory effects of *Citrus unshiu pericarpium* extracts on palmitate-induced lipotoxicity in HepG2 cells

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Abstract *Citrus unshiu pericarpium* (CP), a rich source of flavonoids, has been used as a traditional medicine for purposes of exhaustion, cold, and cancer treatment in East Asia including China and Korea. This study investigated the effect of CP extracts on palmitate-induced lipotoxicity in hepatocytes, HepG2. Cell viability and apoptosis were measured by Trypan blue staining and Hoechst staining, respectively. Cellular uptake of free fatty acid was measured by Oil Red O staining. The results show that CP extracts attenuated palmitate-induced intracellular lipid accumulation and lipotoxicity in HepG2 cells. CP extracts also blocked the expression of apo B protein, an apolipoprotein with functional roles in intracellular lipid accumulation. Moreover, CP extracts inhibited the release of the mitochondrial cytochrome c through regulation of apoptosis-related proteins, Bax and Bcl-2. These results demonstrated a novel function of CP extracts in preventing lipotoxicity in HepG2 cells, implicating a promising phytotherapeutic approach for lipo-detoxification.

Keywords: *Citrus unshiu pericarpium*, palmitic acid, HepG2 cells, lipotoxicity, apoptosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) involves abnormal lipid accumulation in the liver and, is related to metabolic syndrome, a combination of medical disorders such as obesity, insulin resistance, type II diabetes, hypertension and hyperlipidemia. The disease can encompass simple lipids in the liver as well as a non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (1,2). Interest in NAFLD has been increased over recent years because of its high prevalence, which resulted from the increase of metabolic syndrome attributed to nutritional imbalances caused by changes in human behavior and lifestyle (3,4). However, the causative mechanism of NAFLD are still unknown. According to a previously reported hypothesis, free fatty acids that enter into the liver are oxidized to produce energy or stored as triglycerides from excess free fatty acids. Free fatty acids are the principal contributor to lipoapoptosis and lipotoxicity because they are potential cellular toxins and lead to excessive accumulation of lipid (5). Furthermore, it has been reported that individual fatty acids have a inherent toxic potential. The toxic effects of free fatty acid are known to depend on their chain length and degree of saturation. In particular, palmitic acid is the most affluent saturated fatty acid in the blood stream and is known to induce cellular dysfunction and cell death in a number of cell types (6,7). The lipotoxic effect of palmitate appears predominantly when cells from nonadipose tissues are chronically exposed to high

levels of palmitate (8). Thus, the therapy based on reducing lipid accumulation is ideal for treating the NAFLD because accumulation of neutral lipids (triglycerides) in the liver is the main cause for NAFLD (9).

Flavonoids are the members of a large class of natural polyphenolic compounds containing in fruits and vegetables regularly consumed by humans (10). It has been reported that functional phytochemicals such as polyphenols from plants may act as antioxidants to protect against oxidative stress. Given this information, various researches are underway to find natural sources of antioxidants that can be used as therapeutic agents against the pathogenesis of chronic diseases such as cardiovascular, cancer and liver disease (11,12).

Citrus fruits, including orange, grapefruit, lemon, lime, mandarin, and tangerine, are rich in flavonoids and contain several kinds of chemopreventive agents such as glycosides and limonoids, hesperidin, glyceroglycolipids, and *d*-limonene. *Citrus unshiu* (Satsuma mandarin), a seedless and easy peeled citrus fruit of the *Rutaceae* family, is a candidate as natural antioxidant source, as well as a variety of bioactive compounds with medicinal properties have been isolated from them. The peel of such fruits has been used as a traditional herbal medicine in east Asia including Korea and China for treating cancer, muscle tension, nausea, dyspepsia, coughs and phlegm, etc (8). *In vitro* studies have demonstrated that citrus flavonoids may lower blood cholesterol and triglycerides, as demonstrated in a number of animal studies using rabbits, rat, mice, and hamsters (13).

Regardless of its physiological properties, a majority of the citrus peel is thrown away causing an environmental problem. Therefore, it is beneficial to find various applications of citrus peel (CP) in many types of processed foods. In this study, we investigated the effects of CP extracts on palmitate-induced steatosis in hepatocytes and the potential mechanisms underlying protective effects. In order to assess this effect, apoptosis-associated signal transduction pathways and expression of apolipoprotein related to intracellular lipid accumulation were assayed.

Materials and Methods

Preparations of extracts Citrus fruits, which were purchased in a local market located at Jeonju, Korea, were peeled after washing them. Water extracts of CP (CPWE) and ethanol extracts of CP (CPEE) were prepared. Briefly, the CP was dried at room temperature for about 5 days and then blended to make powder. For the CP water extraction, 10 volumes of distilled water were poured into powder, and then heated using an electronic slow boiler for 3 h, followed by filtration and freeze-drying (Ilshin, Yanju, Korea). For the CP ethanol extraction, 10 volumes of 95% ethanol were poured into powder, and then refluxed at 50°C using a condenser, followed by filtration (Whatman, Little Chalfont, UK) and concentrating using a rotary evaporator (EYELA, Tokyo, Japan). The concentrations of CP extracts were prepared by dissolving 20 mg of extracts in 1 mL of distilled water or 95% ethanol.

Reagents Penicillin G, L-glutamine, trypan blue, ethanol, Oil Red O, and palmitate were from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Bcl-2, cytochrome c, caspase-3, apoA1, apo B, and secondary antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA). tBid, cleaved caspase-8, 9, b-actin, and Bax antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). H₂DCF-DA (2',7'-dichlorodihydrofluorescein diacetate, acetyl ester) was sourced from Invitrogen (Carlsbad, CA, USA).

HPLC analysis HPLC analysis was carried out using a Shimadzu SCL-10AVP system controller (Shimadzu, Kyoto, Japan.) with an YMC-Pack ODS-A column (4.6 mmx150 mm I.D., 3 µm particle size, YMC, Kyoto, Japan). The mobile phase was consisted of water/ acetonitrile 77:23 (v/v), the flow rate was 0.7 mL/min, and the separation temperature was 30°C. The UV detector was set at 280 nm. The injection volume was 10 µL.

Preparation of palmitate/BSA complexes Palmitate was saponified with NaOH after dissolving in ethanol. The sodium salt was dried, resuspended in saline and dissolved at 70°C. While the solution was still warm, 20% (w/v) BSA was added and the mixture was stirred at room temperature for 4 h to allow palmitate to bind to albumin. The palmitate/BSA complex (6.4 mmol/L fatty acid: 0.8 mmol/L BSA;

molar ratio, 7:1) was sterilized by filtering, and aliquoted for future use.

Cell cultures and viability assay HepG2 human hepatocarcinoma cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured with a Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing penicillin G (100 U/mL), L-glutamine (2 mM), and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco BRL) at 37°C. At 70% confluence, cells were maintained in serum depleted conditions prior to experiments. The HepG2 cells viability assay was performed using trypan blue staining.

DNA fragmentation assay HepG2 cells were lysed using the lysis buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100] on ice for 10 min. The lysates were applied to a filter tube using a glass fiber fleece followed by centrifugation (at 12,000×g for 30 min). Residual impurities were removed by washing with PBS (phosphate buffered saline), and subsequently DNA was eluted. Samples were analyzed by electrophoresis on a 1% agarose/ Tris-acetate-EDTA (TAE) gel. After electrophoresis, the gel was stained with ethidium bromide and viewed under ultraviolet light to visualize the DNA bands.

Preparation of the mitochondrial/cytosol fraction Subcellular fractionation was performed using the mitochondria isolation kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) was used to measure proteins in subcellular fractions. Cytochrome c was detected by Western blotting.

Western blot analysis HepG2 cells were lysed in 1% Nonidet P-40, 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 100 mM NaF. The lysates were normalized for analyzing total protein content by using a BCA kit (Pierce Biotechnology, Inc.). Equal amounts of protein were mixed with 2x Laemmli loading buffer and preheated at 95°C for 5 min. Samples were then electrophoresed on 10% SDS-polyacrylamide gels followed by transferring to polyvinylidene-difluoride (PVDF) membrane. The membranes were blocked with 5% skim milk and incubated with primary antibodies. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence substrate kit (Pierce Biotechnology, Inc.) were used to detect specific proteins.

Hoechst 33258 staining Morphological changes of cells were assessed by fluorescent microscopy using Hoechst 33258 staining. Fixed cells were stained with Hoechst 33258 (5 µg/mL) at room temperature for 10 min and rinsed, dried, and examined using fluorescence microscope (Olympus, Tokyo, Japan).

Caspase 3 activity assay The activity of caspase-3 was determined using the caspase-3 ELISA kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's protocols. Caspase-3 activity was normalized to cell lysate protein concentration.

Oil red O staining For quantification of intracellular lipid accumulation, the cells were seeded into 24-well plates followed by washing twice with cold PBS and fixed in 3.7% paraformaldehyde for 15 min. Cells were stained with a diluted 0.3% Oil red O solution for 1 h. The excess stain was removed and cells were washed with water. Staining was observed using light microscopy (Olympus).

Determination of total lipid contents The contents of triglyceride (TG) and total cholesterol (TC) were determined enzymatically using commercially available kit (Asan Pharmaceutical, Seoul, Korea). Total lipids in cells and medium were extracted and purified using the method described by Bligh and Dyer (11).

Statistical analysis Data represent the means $\pm$ standard error (SEM) of at least three separate experiments. Statistical analysis was determined using ANOVA. A value of $p < 0.05$ was considered as significant.

Results and Discussion

Analysis of flavonoids in CP extracts Citrus peels contain high concentration of flavonoids in the forms of flavonone glycosides such as narirutin, naringin, hesperidin, and neohesperidin (14,15). These flavonoids are well-known to have health-promoting properties such as antioxidant, anticancer, anti-inflammatory, cardiovascular, and antihyperglycemic activities (16-19). Figure 1 shows the HPLC chromatograms of standard flavonoids, CPWE, and CPEE. Narirutin and hesperidin were two major flavonone glycosides detected in both CPWE and CPEE, whereas naringin and neohesperidin were not detected. The amount (36.45 ± 0.34 mg/g) of hesperidin in CPEE was much higher than that (11.32 ± 0.15 mg/g) of CPWE, whereas narirutin contents in CPWE and CPEE were 15.37 ± 0.05 and 15.77 ± 0.02 mg/g, respectively.

Effects of CP extracts on palmitate-induced cytotoxicity in HepG2 cells In the present study, we characterized the effects of CP extracts on *in vitro* hepatic model of cellular steatosis with lipid over-accumulation profile induced by palmitate. The patients with non-alcoholic fatty liver disease are shown to be increased lipolysis and carried the free fatty acids (FFAs) to the liver. Excessive accumulations

Fig. 1. HPLC chromatograms of compounds isolated from CP extracts. (A) Chromatograms for standard flavonoids. (B) CPWE. (C) CPEE. Peaks: 1, narirutin; 2, naringin; 3, hesperidin; 4, neohesperidin.

Fig. 2. The effect of CP extracts against palmitate-induced cytotoxicity in HepG2 cells. (A) HepG2 cells were treated with 250 μ M palmitate for 60 h. (B, and C) HepG2 cells were pre-treated with various concentration of CP extracts (10, 50, 100, and 250 μ g/mL) for 2 h, then stimulated with 250 μ M palmitate for 24 h and (D) 72 h. Cell viability was determined by trypan blue staining. Data represent the mean $\pm$ SEM of at least three independent experiments. * p >0.05 versus control. # p <0.05 versus palmitate. Con, control; Pal, Palmitate; CPWE, water extract of citrus unshiu pericarpium; CPEE, ethanol extract of citrus unshiu pericarpium.

of FFAs are associated with more serious liver disease. Many papers have shown that saturated long-chain fatty acids are the major factor of lipotoxicity. The main fatty acids in the human body are palmitic and oleic acids. They are widely used *in vitro* studies to induce steatosis in cultured human liver cell lines or primary cells as well as steatosis stimulated *in vivo* (20).

The potential cytotoxic effect of palmitate or CP extracts in HepG2 cells was first examined. As shown in Fig. 2A, treatment with 250 μ M palmitate for 60 h decreased the survival of HepG2 cells which was time-dependently. In contrast, treatment with CPWE or CPEE (10–250 μ g/mL) did not affect the cell viability (data not shown). Given these findings, we examined the effects of CP extracts on palmitate-induced cytotoxicity in HepG2 cells. As shown in Fig. 2B and 2C, pretreatment of HepG2 cells with CPWE and CPEE reduced palmitate-induced cytotoxicity in a dose-dependent manner, showing a similar pattern between two CP extracts. Moreover, HepG2 cells pretreated with 100 μ g/mL CPWE or CPEE significantly blocked palmitate-induced cell death up to 60 h incubation, while the cell viability of palmitate-induced cells was gradually decreased with time (Fig. 2D). Based on these results, we chose 100 μ g/mL CPWE and CPEE and 250 μ M palmitate as the optimal treatment concentration throughout the study.

Effects of CP extracts on palmitate-induced lipid accumulation in HepG2 cells

Palmitate, a saturated long-chain fatty acid, has also

been known to induce apoptosis in various cell types (8), including cardiomyocytes, pancreatic b-cells, haematopoietic cells, and astrocytes. Although the mechanism by which palmitate induces cell damage and cell death is not yet fully known, reactive oxygen species (ROS) and cytochrome c have been identified as two major intracellular mediators of palmitate-induced cell death (21,22). In this study, we demonstrated the inhibitory effects of CP extracts using *in vitro* steatosis models of HepG2 cells. In order to investigate the inhibitory effects of CP extracts, the HepG2 cells were treated with the addition of palmitate.

The effect of CP extracts on palmitate-induced lipid accumulation was measured by using Oil Red O staining. As shown in Fig. 3A, lipid droplets were observed in palmitate-treated cells. The staining of lipid droplets was relatively weaker in CP extracts-treated cells than those treated with palmitate. Therefore, the degree of lipid accumulation within the cell and medium was measured as triglycerides and total cholesterol (TC). As shown in Fig. 3B and 3C, treatment of palmitate significantly increased intracellular triglycerides and TC levels, whereas pretreatment of HepG2 cells with CPWE and CPEE significantly decreased intracellular triglycerides and TC levels, showing a similar pattern between two CP extracts. Furthermore, pre-incubation of HepG2 cells with 100 μ g/mL CPWE or CPEE significantly blocked palmitate-induced lipid accumulation for 24 h (Fig. 3D). We confirmed accumulation of palmitate-induced triglyceride and TC in HepG2 cell was reduced by the addition of CP extracts. From morphological

Fig. 3. The effect of CP extracts against palmitate-induced lipid deposition in HepG2 cells. Cells were pre-treated with 100 $\mu\text{g}/\text{mL}$ of CP extracts for 2 h, and treated with 250 μM palmitate for 24 h. (A) Photomicrograph shows Oil Red O staining in HepG2 cells. After incubation for 24 h, cells were stained with Oil Red O. (B) Quantitative analysis of lipid accumulation as total cholesterol and (C) triglyceride in lysate and medium of HepG2 cells. (D) Quantitative analysis of intracellular total cholesterol and triglyceride as a function of time. Data represent the mean $\pm$ SEM of at least three independent experiments. * $p > 0.05$ versus control. # $p < 0.05$ versus palmitate. Control, untreated; CPWE & CPEE, treated with 100 $\mu\text{g}/\text{mL}$; palmitate, 250 μM ; Pal+CPWE, 250 μM palmitate+100 $\mu\text{g}/\text{mL}$ CPEE.

Fig. 4. The effect of CP extracts on the production of lipoproteins related to lipid accumulation apo A1 and apo B in HepG2 cells. Proteins related to lipid accumulation were assayed using western blots. Western blot analysis of each protein of b-actin and CBB were used to confirm the equal amounts of protein used for electrophoresis. Data are presented as means $\pm$ SEM of three independent experiments. * p <0.05 versus control, # p <0.05 versus palmitate. Control, untreated; CPWE & CPEE, treated with 100 μ g/mL; palmitate, 250 μ M; Pal+CPWE, 250 μ M palmitate+100 μ g/mL CPEE.

observation and quantitative analysis, we also found palmitate-induced lipid accumulation in HepG2 cell was decreased time dependently after treatment of cells with CP extracts.

Effects of CP extracts on palmitate-induced production of lipid absorption-related apolipoproteins, apo A1 and B Increased levels of plasma triglyceride and cholesterol are associated with the development of metabolic diseases. Very-low-density lipoprotein (VLDL) secreted from hepatocytes contains a hydrophobic core consisted of TG, cholesterol esters, phospholipids, and apo B. Secretion of VLDL plays a very important role in controlling the plasma levels of TG and cholesterol (23,24). Based on the above findings, we examined the effects of CP extracts on palmitate-induced production of lipid absorption-related lipoproteins, apoA1 and B. Figure 4 shows the expression levels of apoA1 and apoB in the lysate and medium. CP extracts significantly decreased palmitate-induced expression level of ApoB in the lysate, but increased ApoB level in the medium. Neither palmitate nor CP extracts altered the expression of ApoA1 in both lysate and medium.

Our present results confirm that CP extracts decreased the secretion of apo B lipoprotein regardless of the intracellular lipogenic state. The synthesis of apo B occurs primarily in hepatic and intestinal cells. When apo B metabolism is used as a nutritional material from the

nutritional point of view, it is meaningful to check degradation, assembly, and secretion of VLDL in cultured hepatocytes. The overproduction of apo B-containing lipoproteins has been recognized as a mechanism underlying coronary artery disease and hyperlipidemia (25,26).

Effects of CP extracts on palmitate-induced apoptosis in HepG2 cells

More recently extensive evidences have been reported that hepatic lipotoxicity is associated with apoptosis (27,28). We have demonstrated that CP extracts showed anti-apoptotic effects against palmitate-induced lipoapoptosis in HepG2 cells. The protective effect of CP extracts on palmitate-induced apoptosis was examined using Hoechst 33258 staining. Significant morphological changes in the nuclear chromatin were observed in cells treated with palmitate (Fig. 5A). The blue emission light was brighter in palmitate-treated cells than control cell. In contrast, nuclei of CP extracts-treated cells showed lesser bright blue staining than palmitate-treated cells. In addition, the cellular DNA fragmentation was observed clearly in HepG2 cells treated with palmitate, while there was no effect in pretreated cells with CP extracts (Fig. 5B). Therefore, we determined Caspase-3 activity in HepG2 cells treated with palmitate to examine whether there is any anti-apoptotic effect of CP extracts which is mediated through mitochondria-dependent pathway. As a resultant,

Fig. 5. The effects of CP extracts against palmitate-induced apoptosis in HepG2 cells. (A) Fluorescence photomicrograph of HepG2 cells stained with Hoechst 33258. Cells were pre-treated with 100 $\mu\text{g/mL}$ of CP extracts for 2 h and treated with 250 μM palmitate for 24 h. (B) Photograph of UV-illuminated DNA from harvested cells resolved on 1% agarose gels by electrophoresis and stained with ethidium bromide. (C) The activity of caspase-3 was induced by 8 h palmitate treatment in HepG2 cells. Caspase activity of control cells were defined as 1-fold. Data are presented as means $\pm$ SEM of three independent experiment. * $p < 0.05$ versus control, # $p < 0.05$ versus palmitate. Control, untreated; CPWE & CPEE, treated with 100 $\mu\text{g/mL}$; palmitate, 250 μM ; Pal+CPWE, 250 μM palmitate+100 $\mu\text{g/mL}$ CPEE.

CP extracts pretreatment significantly reduced palmitate-induced caspase-3 activity (Fig. 5C).

To investigate the potential mechanism for the protective effects of CP extracts on palmitate-induced apoptosis, the expression of apoptotic proteins was measured by Western blotting. As shown in Fig. 6A, treatment with palmitate remarkably increased Bax expression but decreased Bcl-2 expression, whereas treatment of cells with CP

extracts significantly decreased Bax expression but increased Bcl-2 expression. Moreover, the treatment of CP extracts decreased the expressions of cleaved-8, 9 and tBid. The CP extracts also inhibited the release of cytochrome c from the mitochondria into the cytosol (Fig. 6B).

We first confirmed that palmitate-induced lipooptosis was blocked in CP extracts treated HepG2 cell through Hoechst staining and DNA fragmentation. Also, CP extracts inhibited the release of the mitochondrial cytochrome c through regulation of apoptosis-related proteins, Bax and Bcl-2. These findings suggest that CP extracts regulate mitochondrial cell death pathway in palmitate-treated condition. As a resultant, hepatocyte lipotoxicity involves a complex network of proapoptotic stimuli (29). These results are consistent with recent reports, in which stearic acids, saturated fatty acids, and palmitic in hepatocytes, lead to both time and concentration-dependent lipooptosis (6). These toxic fatty acids stimulate the apoptotic intrinsic pathway, causing to Bax activation, mitochondrial permeabilization, release of cytochrome c, and activation of caspase-3 and 7 (30).

In conclusion, our findings demonstrated the protective effects of CP extracts on palmitate-induced steatosis via suppression of Apo B protein in HepG2 cells. These results suggest that CP extracts are effective agents for protecting the liver cells against palmitate-induced steatosis and can help to treat fatty liver diseases like NASH.

Disclosure The authors declare no conflict of interest.

References

- Patton HM, Sirlin C, Behling C, Middleton M, Schwimmer JB, Lavine JE. Pediatric nonalcoholic fatty liver disease: A critical appraisal of current data and implications for future research. *J. Pediatr. Gastr. Nutr.* 43: 413-427 (2006)
- Utzschneider KM, Kahn SE. The role of insulin resistance in nonalcoholic fatty liver disease. *J. Clin. Endocr. Metab.* 91: 4753-4761 (2006)
- Pangso G, Pancini G, Musso G, Gambio R, Mecca F, Depetris N, Cassader M, David E, Cavallo-Perin P, Rizzetto M. Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: Further evidence for an etiologic association. *Hepatology* 35: 367-372 (2002)
- Bugianesi E, McCulloch AJ, Marchesini G. Insulin resistance: A metabolic pathway to chronic liver disease. *Hepatology* 42: 987-1000 (2005)
- Wang D, Wei Y, Pagliassotti MJ. Saturated fatty acids promote endoplasmic reticulum stress and liver injury in rats with hepatic steatosis. *Endocrinology* 147: 943-951 (2006)
- Malhi M, Bronk SF, Werneburg NW, Gores GJ. Free fatty acids induce JNK-dependent hepatocyte lipooptosis. *J. Biol. Chem.* 281: 12093-12101 (2006)
- Eduardo R, Leonor RS, Eunat GV, Pilar Z, Eva M, Mar GB. Lipotoxicity, fatty acid uncoupling and mitochondrial carrier function. *Biochim. Biophys. Acta* 1797: 800-806 (2010)
- Lu Y, Zang C, Bucheli P, Wei G. Citrus flavonoids in fruit and traditional Chinese medicinal food ingredients in china. *Plant Food Hum. Nutr.* 61: 57-65 (2006)
- Yochum L, Kushi L, Meyer K, Folsom AR. Dietary flavonoid intake and risk of cardiovascular disease in postmenopausal women. *Am. J. Epidemiol.* 149: 943-949 (1999)
- Kanno S, Tomizawa A, Hiura T, Osanai Y, Shouji A, Ujibe M, Ohtake T, Kimura K, Ishikawa M. Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. *Biol. Pharm. Bull.* 28: 527-530 (2005)
- Boyer J, Liu RH. Apple phytochemicals and their health benefits. *Nutr. J.* 3: 1-15 (2004)
- Dai J, Mumper RJ. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15: 7313-7352 (2010)

Fig. 6. Effects of CP extracts on palmitate-induced expression of apoptosis related proteins in HepG2 cells. Cells were incubated for 24 h. (A) Expressions of apoptosis-related proteins were determined by western blots. Protein levels were determined relative to untreated control cells after normalization to β -actin. (B) Markers of mitochondria apoptotic pathway were measured by western blotting. The quantitative analyses of apoptotic protein expression are displayed in the mitochondria and cytosol. Data are presented as means $\pm$ SEM of three independent experiments. * p <0.05 versus control, # p <0.05 versus palmitate. Control, untreated; CPWE & CPEE, treated with 100 μ g/mL; palmitate, 250 μ M; Pal+CPWE, 250 μ M palmitate+100 μ g/mL CPEE.

13. Lin Y, Vermeer MA, Bos W, Van Buren L, Schuurbiens E, Miret-Catalan S, Trautwein EA. Molecular structures of citrus flavonoids determine their effects on lipid metabolism in HepG2 cells by primarily suppressing apo B secretion. *J. Agr. Food Chem.* 59: 4496-4503 (2011)
14. Rouseff RL, Martin SF, Youtsey CO. Quantitative survey of narirutin, naringin, hesperidin, and neohesperidin in citrus. *J. Agr. Food Chem.* 35: 1027-1030 (1987)
15. Bocco A, Cuvelier ME, Richard H, Berset C. Antioxidant activity and phenolic composition of citrus peel and seed extracts. *J. Agr. Food Chem.* 46: 2123-2129 (1998)
16. Yu J, Wang L, Walzem RL, Miller EG, Pile LM, Patil BS. Antioxidant activity of citrus limonoids, flavonoids, and coumarins. *J. Agr. Food Chem.* 53: 2009-2014 (2005)

- (2005)
17. Garcia BO, Castillo J. Update on uses and properties of citrus flavonoids: New findings in anticancer, cardiovascular, and anti-inflammatory activity. *J. Agr. Food Chem.* 56: 6185-6205 (2008)
 18. Lee SG, Ra JH, Song JY, Gwak CH, Kwon HJ, Yim SV, Hong SP, Kim JJ, Lee KH, Cho JJ, Park YS, Park CS, Ahn HJ. Extracts from *Citrus unshiu* promote immune-mediated inhibition of tumor growth in a murine renal cell carcinoma model. *J. Ethnopharmacol.* 133: 973-979 (2011)
 19. Shen W, Xu Y, Lu YH. Inhibitory effects of Citrus flavonoids on starch digestion and antihyperglycemic effects in HepG2 cells. *J. Agr. Food Chem.* 60: 9609-9619 (2012)
 20. Nakamura S, Takamura T, Matsuzawa-Nagata, N, Takayama H, Misu H, Noda H, Nabemoto S, Kurita S, Ota T, Ando H, Mitamoto KI, Kaneko S. Palmitate induces insulin resistance in H4IIEC3 hepatocytes through reactive oxygen species produced by mitochondria. *J. Biol Chem.* 284: 14809-14818 (2009)
 21. Bligh EG, Dyer WJ. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Phys.* 37: 911-917 (1959)
 22. Dörmann P, Voelker TA, Ohlrogge JB. Accumulation of palmitate in *Arabidopsis* mediated by the acyl-acyl carrier protein thioesterase FATB1. *Plant Physiol.* 123: 637-643 (2000)
 23. Listenberger LL, Han X, Lewis SE, Cases S, Farese Jr RV, Ory DS, Schaffer JE. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Pans* 100: 3077-3082 (2003)
 24. Davis, RA. Cell and molecular biology of the assembly and secretion of apolipoprotein B-containing lipoproteins by the liver. *Biochim. Biophys. Acta* 1440: 1-31 (1999)
 25. Tachobana S, Sato K, Cho Y, Chiba T, Schneider WJ, Akiba Y. Octanoate reduces very low-density lipoprotein secretion by decreasing the synthesis of apolipoprotein B in primary cultures of chicken hepatocytes. *Biochim. Biophys. Acta* 1737: 36-43 (2005)
 26. Arrol S, Mackness MI, Durrington PN. The effects of fatty acids on apolipoprotein B secretion by human hepatoma cell (HepG2). *Atherosclerosis* 159: 255-264 (1999)
 27. Scott MG. Obesity, metabolic syndrome, and cardiovascular disease. *J. Clin. Endocr. Metab.* 89: 2595-2600 (2004)
 28. Kakisaka K, Cazanave SC, Werneburg NW, Razumilava A, Mertens JC, Bronk SF, Gores GJ. A hedgehog survival pathway in 'nudead' lipotoxic hepatocytes. *J. Hepatol.* 57: 844-851 (2012)
 29. Wieckowska A, Zein NN, Yerian LM. *In vivo* assessment of liver cell apoptosis as a novel biomarker of disease severity in nonalcoholic fatty liver disease. *Hepatology* 44: 27-33 (2006)
 30. Matthew JW, Gregory RS. Regulation and function of triacylglycerol lipases in cellular metabolism. *Biochem. J.* 414: 313-325 (2008)