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Antioxidant and anti-inflammatory effects of Chinese propolis during palmitic acid-induced lipotoxicity in cultured hepatocytes

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ABSTRACT

Overload of hepatic palmitate, like palmitic acid (PA), is known as the key trigger for non-alcoholic fatty liver disease. Here, we investigated whether Chinese propolis (CP) would mitigate PA-induced lipotoxicity in hepatocytes. Total of 20 phenolic compounds were analysed in CP using HPLC-DAD/Q-TOF-MS. PA has significant cytotoxic effects on HepG2 and L02 hepatocytes, by decreasing cell viability and inducing LDH releases, which was rescued by CP pre-treatment. Lipoapoptosis was also found in HepG2 hepatocytes treated with PA and CP helped restore the energy provision and prevented cell apoptosis. After PA challenges, antioxidant effects of CP were also observed by boosting the HepG2 cellular total-antioxidant potentials, increasing superoxide dismutase level and up-regulating antioxidant/detoxicant gene expressions (*GSTA1*, *TXNRD1*, *NQO-1*, *HO-1* and *Nrf2*). Notably, gene expressions of inflammatory cytokine, TNF-α and IL-8, were decreased by CP. Therefore, CP protects hepatocytes against PA-induced lipotoxicity by lowering biomarkers of apoptosis, oxidative stress and inflammation.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD), a globally prevalent serious liver disease, is commonly characterized by the accumulation of the fat in the liver (Cheung & Sanyal, 2010). NAFLD is currently recognized as a clinical feature of metabolic syndrome, and it is usually accompanied by other metabolic disorders, such as obesity, cardiovascular disease and type 2 diabetes mellitus (Vaidecantos et al., 2015). Most patients with NAFLD only have benign steatosis. Nevertheless, 5–10% of NAFLD cases are associated with non-alcoholic steatohepatitis (NASH), which involves hepatic inflammation, hepatocellular damage and liver fibrosis. NASH patients also have a high risk of terminal liver failure, hepatocirrhosis, portal hypertension and hepatocellular carcinoma (Vanni et al., 2010).

Although the pathogenesis of NASH is complicated and still not fully understood, Day and James raised a prevailing "two hits" hypothesis for NASH pathogenesis (Day & James, 1998). The "first" hit is the overload of the free fatty acids (FFAs) and triacylglycerol, which is produced by a metabolic disturbance in the hepatocytes, leading to steatosis. The lipid oxidation will lead to increased levels of reactive oxygen species (ROS), which may induce cellular apoptosis and inflammation. Therefore, the "second" hit involves some other factors that accompany the steatosis, such as ROS-mediated oxidative stress, decreased ATP production and overproduction of proinflammatory cytokines (like TNF-α, interleukins, etc.). Currently, there is no effective therapeutic strategy for NAFLD or NASH (Van Wagner & Rinella, 2011). Even the most promising candidate drugs have significant adverse effects (Singh, Khera, Allen, Murad, & Loomba, 2015). Hence, the development of a novel drug for treating NAFLD and NASH is urgently needed.

Recently, substantial attention has been paid to suppress hepatocytes oxidative stress and to attenuate cellular inflammation in NAFLD and NASH therapeutic strategy development (Nabavi, Rafraf, Somi, Homayouni-Rad, & Asghari-Jafarabadi, 2015; Rolo, Teodoro, & Palmeira, 2012). Several studies have shown that imbalances in ROS can be restored through boosting the cellular antioxidant potential and increasing the cellular detoxifying enzymes (Takahashi et al., 2014). Nuclear factor erythroid-2-related factor 2 (Nrf2) is known as the "master" modulator by binding to antioxidant response elements (AREs) and activating detoxifying enzymes in different cell types (Jin et al., 2016). In hepatocytes, Nrf2 suppresses the expression of several genes that are related to cell metabolism/fatty acid (FA) synthesis, which

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highlights its important role in NASH development (Kitteringham et al., 2010). Moreover, ROS activate nuclear factor (NF)-κB, which facilitate the synthesis of several proinflammatory cytokines and exacerbate the necroinflammation and fibrosis of the liver (Rolo, Teodoro, & Palmeira, 2012). Therefore, natural antioxidant and anti-inflammatory reagents, particularly some nutrients from dietary origins, have been widely investigated for their potential hepatoprotective properties (Farghali, Canova, & Zakhari, 2015; Masterjohn & Bruno, 2012). These regents have been shown to protect the liver from ROS accumulation/oxidative stress during FA oxidation in the liver and they suppress lipotoxicity-induced hepatocyte inflammation.

Propolis is an emerging health product that is gathered by honeybees from various plant sources. It has well-documented hepatoprotective activity both *in vitro* and *in vivo* (Banskota et al., 2000; Paulino, Barbosa, Paulino, & Marcucci, 2014). Nevertheless, the potential effects and hepatocellular effects/mechanisms of propolis against NAFLD and NASH remain unknown. Here, we investigated the effects of Chinese propolis (CP) in preventing palmitic acid (PA), a representative long-chain saturated FFA, which induced lipotoxicity in cultured hepatocytes.

2. Materials and methods

2.1. Reagents

HPLC-grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). The ultrapure water was obtained from a Millipore Milli-Q water purification system (Millipore, Bedford, MA, USA). Absolute alcohol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). The standards of 20 phenolic compounds used for HPLC-DAD/Q-TOF-MS analysis were obtained from Sigma-Aldrich (St. Louis, Mo., USA). All other reagents were obtained from Sangon Biotechnology (Shanghai, China) or as indicated in the specified methods.

2.2. Sample preparation

The standards of the 20 phenolic compounds were dissolved in methanol and prepared for the mixed standard stock solution; then, they were diluted to a series of working standard solutions with different concentrations for further working curve construction. The Chinese propolis sample was obtained from the Hebei

Table 1
HPLC-DAD/Q-TOF-MS analysis on Chinese propolis.

Compounds	RT (min)	$[M+1]^+$	Content (mg/g)	Compounds	RT (min)	$[M+1]^+$	Content (mg/g)
Vanillic Acid	7.784	169.0495	0.833	Caffeic acid	8.709	181.0495	8.024
p-Coumaric acid	13.748	165.0546	1.828	Ferulic acid	16.065	195.0652	1.887
trans-Isoferulic acid	17.522	195.0652	2.572	Rutin	20.246	611.1607	/
3,4-Dimethoxycinnamic acid	20.748	209.0808	6.148	Myricetin	21.056	319.0448	2.731
Morin	22.109	303.0499	/	Cinnamic acid	22.142	149.0597	0.297
Quercetin	23.001	303.0499	0.626	Pinobanksin	23.195	273.0757	7.358
Luteolin	23.730	287.0550	56.513	Kaempferol	24.491	287.0550	1.856
Galangin	24.961	271.0601	7.971	Pinocembrin	26.326	257.0808	7.164
CAPE	27.104	285.1121	47.523	Chrysin	27.181	255.0652	18.176
Apigenin	27.376	271.0601	16.547	Curcumin	27.554	369.1333	0.020

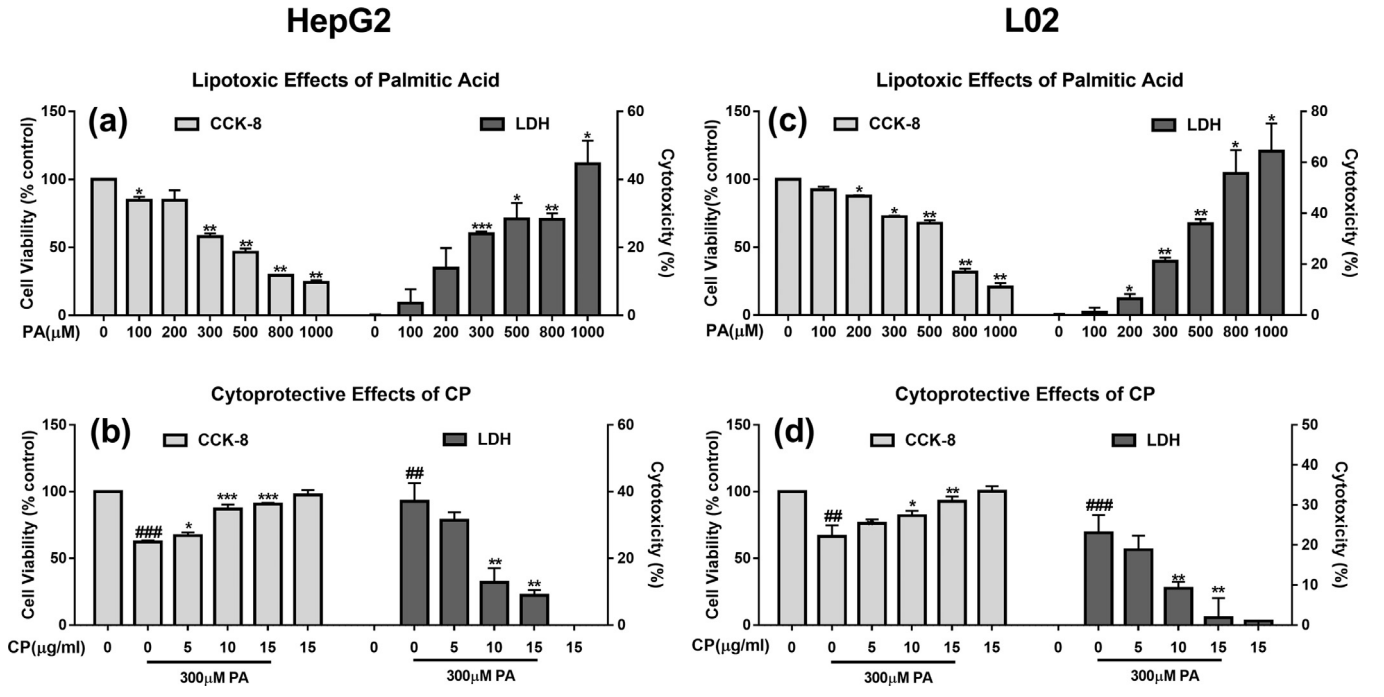

Fig. 1. Chinese propolis prevents palmitic acid-induced lipotoxic effects in HepG2 and L02 hepatocytes. Increased concentrations of palmitic acid (PA) were treated in HepG2 (a) or L02 (c) cells for 12 h. Lipotoxicity was determined with cell counting kit (CCK)-8 assays and LDH (lactate dehydrogenase) tests. Data are shown as the mean ± SD from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the untreated cells. HepG2 (b) or L02 (d) cells were pre-treated with various concentrations of CP followed by 12 h of PA (300 μM) treatment. CCK-8 assays and LDH tests were used to determine the lipotoxicity. Data are shown as the mean ± SD from three independent experiments. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ indicate significant differences from untreated cells. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences from PA-treated cells.

province of China in the summer of 2016 (voucher specimen no. CP160628). Then the sample was weighed and extracted by 95% ethanol, and they were then agitated in an ultrasonic water bath for 45 min and filtered. Additionally, the residue was re-extracted twice under the same conditions. After the 3rd extraction, the filtered solutions were combined and dried (Wang et al., 2015). For *in vitro* studies, CP was dissolved in ethanol to get 15 mg/mL stock solutions. The final dry matter was also re-dissolved in methanol to a concentration of 5 mg/mL for HPLC-DAD/Q-TOF-MS chemical analysis.

2.3. HPLC-DAD/Q-TOF-MS analysis

The extracts were separated using an Agilent 1200 series Rapid Resolution LC system (Agilent Technologies, CA, USA) consisting of a vacuum degasser, autosampler, and binary pump. This instrument was equipped with an Agilent Poroshell 120 EC-C18 column (2.1 mm × 100 mm, 2.7 μm) from Agilent Technologies. A gradient elution was programmed using as a mobile phase A, water, and as a mobile phase B, methanol. The programme was: 15% (B) at 0–2 min, 15–30% (B) at 2–10 min, 30–90% (B) at 10–25 min, 90% (B) at 25–30 min, 90–15% (B) at 30–31 min, and 15% (B) at 31–45 min. The flow rate was set at 200 μL/min throughout the gradient. The injection volume was 2 μL, and UV spectra were recorded from 190 to 400 nm using the DAD detector, whereas the chromatograms were registered at 270, 330, and 350 nm.

MS was performed in an Agilent 6510 ESI-Q-TOF (standard 1 GHz). The optimal MS conditions consisted of a capillary voltage of 4.0 kV in positive ionization mode, a skimmer voltage of 65 V, and a fragmentor at 135 V. The gas temperature was 350 °C, the drying gas flow rate was 11 L/min, and the nebulizer pressure was 40 psi. Nitrogen was used as the collision, drying, and nebulizer gas. MS spectra were acquired at 100–3200 m/z at a scan rate of 2.0 spectra/s by varying collision energy with mass. A reference mass solution containing reference ions (121.050873, 322.048321, and 922.009798) was used to maintain the mass accuracy during the run. The Mass Hunter Workstation software (Build 4033.1, Patch One, Agilent, Santa Clara, CA, USA).

2.4. Cell culture and palmitic acid treatment

Human hepatic cell line HepG2 was purchased from Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai (Shanghai, China). Another human hepatic cell line, L02, was a generous gift from Professor Baoping Ji (College of Food Science and Nutritional Engineering, China Agricultural University, China). Cells were grown and maintained in a humidified incubator at 37 °C and 5% CO_2 using high-glucose Dulbecco's modified Eagle's medium (Pierce HyClone, Fremont, CA, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% (V/V) foetal bovine serum, which was obtained from Gibco (Carlsbad, CA, USA). All studies were performed using 70–80% confluent cells before treatment. To induce lipotoxicity, HepG2 or L02 cells were treated with designed concentrations of PA (Sigma-Aldrich, St. Louis, MO, USA) (Nissar, Sharma, & Tasduq, 2015).

2.5. Lipotoxicity determination

The lipotoxicity of PA for the HepG2 and L02 cells was measured by the cell counting kit (CCK-8) assay and lactate dehydrogenase (LDH) activity, respectively. After designed treatments, 10 μL of CCK-8 solution (Dojindo, Kumamoto, Japan) was added into the cells cultured in 96-well plates and OD values at 450 nm were measured using a microplate reader (M5, MD, USA). The LDH activity in the cell culture media was determined by a commercial kit (Beyotime, Haimen, China) according to the manufacturer's instructions. The OD values at 490 nm were measured using a microplate reader.

2.6. Detection of ATP activity

HepG2 cells were cultured in 12-well plates and treated as designed. Then, the cells were washed with chilled PBS twice and lysed with ATP lysis buffer from the ATP assay kit (Beyotime Institute of Biotechnology, China) and centrifuged at 12,000*g* for 5 min at 4 °C. Afterwards, cell supernatant (50 μL) were mixed thoroughly with dilution buffer (50 μL) provided from the kit, containing luciferase which has been warmed at room temperature for 3 min. Luminance was determined by using the M5 microplate reader with a luminometer. ATP levels were then calculated according to the standard curve and normalized based on cellular protein level, measured by the enhanced BCA Protein Assay Kit (Beyotime).

2.7. Caspase 3/7 activity measurement

A hallmark of apoptosis, caspase 3/7 activity was measured using commercially available kits according to the manufacturer's instructions (Caspase 3/7 activity apoptosis assay kit, green fluorescence, Sangon Biotechnology). Briefly, Caspase 3/7 assay loading solution (Caspase 3/7 Substrate, 50 μL and assay buffer, 10 mL) were thawn and mixed completely at room temperature. After designed treatment, assay loading solution were added into 96 well plates (10 μL/well) then incubated at room temperature for 1h. Afterwards, monitor the fluorescence intensity at 530 nm emis-

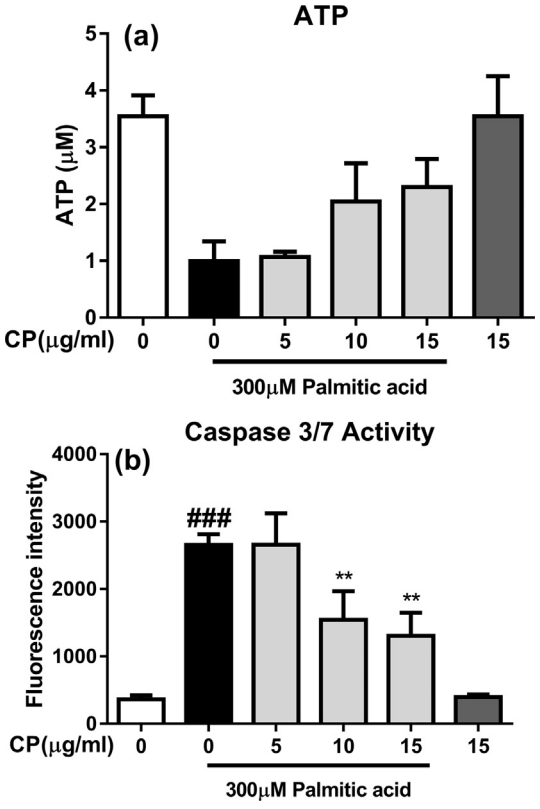

Fig. 2. Chinese propolis protects palmitic acid-induced mitochondrial ATP depletion and lipoapoptosis in HepG2 cells. HepG2 cells were pre-treated with various concentrations of CP followed by 12 h of PA (300 μM) treatment. HepG2 intracellular ATP concentrations (a) and caspase 3/7 activity (b) were measured after 12 h of PA exposure. Data are shown as the mean ± SD from three independent experiments. $^{###}p < 0.001$ means significantly different from untreated cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences from PA-treated cells.

sion and 485 nm excitation using the M5 microplate reader. The fluorescence in blank wells with the growth medium is subtracted from the values for those wells with the cells.

2.8. Cellular antioxidant response measurement

Following appropriate treatments, cells were lysed for the total antioxidant activity (T-AOC) as well as superoxide dismutase (SOD) activities using previous published methods with commercial available kits (Beyotime, Haimen, China). Briefly, T-AOC assay was performed based on ABTS methods. The ABTS working solution was prepared and kept in dark for 12 h before the experiments. Assays were performed in 96 well plates, ABTS working solution (200 μL) were mixed with 10 μL PBS (negative control), trolox standard solution (positive control) or cell lysis, respectively. The OD values at 734 nm were measured using a microplate reader. For SOD assay, the WST-8 colorimetric method was employed following kit's instructions. The OD560 was recorded with microplate reader. One SOD enzymatic activity unit was defined as the amount of sample needed to achieve 50% inhibition of the rate of WST-8 formazan dye reduction. The concentration of total protein was quantified via Enhanced BCA Protein Assay Kit (Beyotime). The SOD activities were the ratio of total enzyme activity unit to the total protein, and the results were expressed as U/mg protein. Each analysis was performed in triplicate.

2.9. Quantitative real-time PCR

As previously described, total cellular RNA was extracted using an RNA Pure Kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) according to the instructions from the manufacturer and was reverse-transcribed into cDNA with a PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) (Jin et al., 2016). Quantitative PCR was performed with SYBR Premix Ex Taq (TaKaRa) using a Real-time PCR Detection System (7500c, Applied Biosystems, Carlsbad, CA, USA). The expression of the housekeeping gene *Gapdh* was used to normalize the expression levels. The primers are designed to flank introns with Primer 5 software (Premier Biosoft, Palo Alto, CA, USA). The specificity of the primers was checked by the melting curve. The PCR products have also been tested by DNA sequencing and electrophoresed on the agarose gel. The primer sets are listed in supplemental Table 1.

2.10. Western blot analysis

Immunoblot analysis were performed as described previously (Jin et al., 2016). Cellular proteins were lysed and equal amounts of protein (20 μg) were separated by SDS–PAGE and then transferred to PVDF membranes, which were incubated with primary antibodies (Rabbit Nrf2 and β-tubulin, Abcam, Cambridge, UK). AP-conjugated anti-rabbit IgG antibody was used as a secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). Bound antibody complexes were visualized using NBT/BCIP solution with colour development buffer. The Western blot results were quantified using Quantity One software.

2.11. Statistical analysis

Data presented as the means ± SD of at least three independent experiments. Statistical differences between two groups were obtained using the Student's *t*-test and one-way analysis of variance (ANOVA) with a post hoc Dunnett test. $P < 0.05$ was accepted as statistically significant.

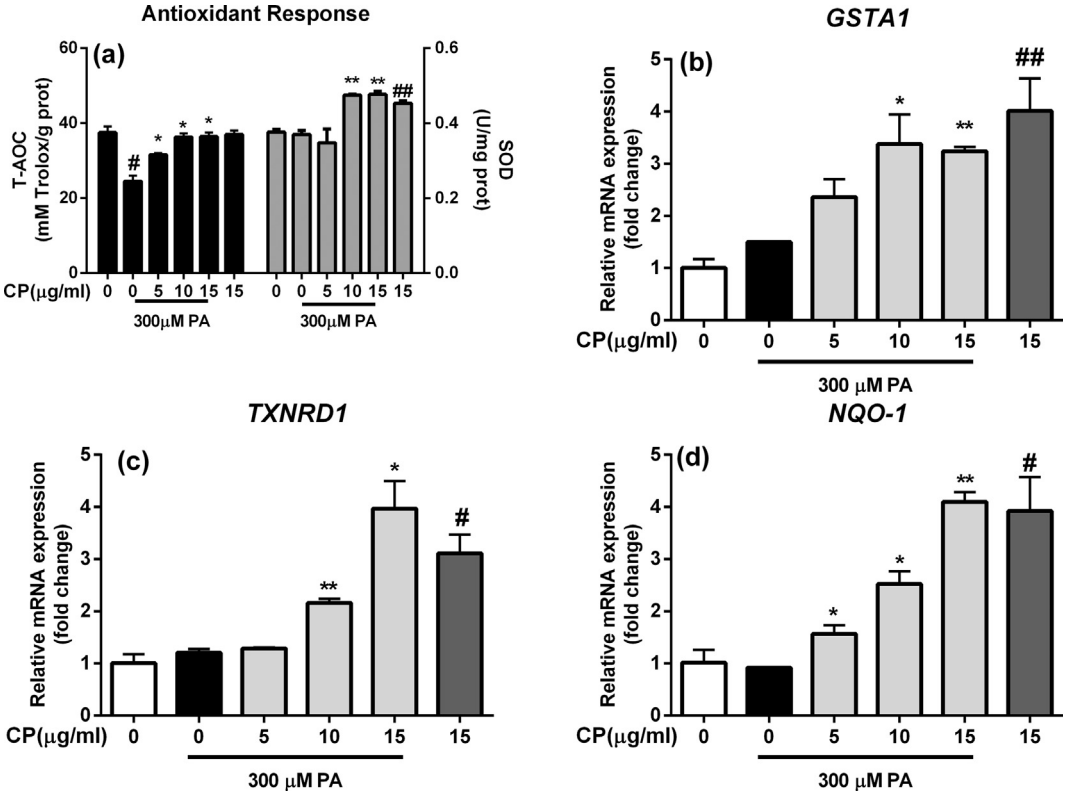

Fig. 3. Effects of Chinese propolis on the cellular antioxidant status in HepG2 cells challenged with palmitic acid. HepG2 cells were pre-treated with various concentrations of Chinese propolis followed by 12 h of PA (300 μM) treatment. (a) HepG2 cellular oxidative stress markers are shown as the T-AOC and SOD levels. (b-d) mRNA expression changes in several important antioxidant/detoxicant genes (*GSTA1*, *TXNRD1* and *NQO-1*). Data are shown as the mean ± SD from three independent experiments. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ indicate significant differences compared to untreated cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences compared to PA-treated cells.

3. Results and discussion

3.1. Phytochemical analysis of Chinese propolis

A total of 20 phenolic compounds were identified and quantified in CP based on HPLC-DAD/Q-TOF-MS analysis (Table 1 and supplemental Table 2). As shown in Table 1, except for rutin and morin, 18 phenolic compounds were detected in CP. Luteolin (56.51 mg/g) and caffeic acid phenyl ester (CAPE, 47.52 mg/g) are the dominant ingredients in CP. According to previous reports, luteolin has antioxidative, anti-inflammatory and anti-ER stress properties in acetaminophen-induced liver injury in mice (Tai et al., 2015). CAPE also has the potential to decrease oxidative stress and hepatic injury caused by acute dichlorvos intoxication (Alp et al., 2016). Additionally, there are a certain number of flavonols in the extracts, such as chrysin (18.18 mg/g) and apigenin (16.55 mg/g), which have known beneficial bio-activities. For example, Huang et al. suggested that chrysin and apigenin can protect against tert-butyl hydroperoxide (tBHP)-induced oxidative stress in rat primary hepatocytes (Huang et al., 2013). Therefore, it can be seen that the rich phenolic compounds in CP contribute to the better bio-activities, as shown in the following research.

3.2. Chinese propolis prevents palmitic acid-induced lipotoxic effects in HepG2 and L02 hepatocytes

Palmitic acid is one representative long-chain saturated FFA that is usually observed with an elevation in obesity and in insulin resistant patients (Bigornia, Lichtenstein, Harris, & Tucker, 2016). Metabolized FFAs (like PA) in the hepatocytes will lead to the release of triacylglycerol, which will accelerate the pathological progression of NAFLD to NASH. As shown in Fig. 1a, high concentrations of PA treatment (12 h) have significant cytotoxic effects on the HepG2 cells, resulting in decreased CCK-8 activity and increased LDH leakage. Compared to previously published literature, pathophysiological concentrations of PA, within 300–500 μM, might cause fat accumulation and lipotoxicity in hepatocytes (Ganji, Kashyap, & Kamanna, 2015; Joshi-Barve et al., 2007). As a result, in our subsequent studies, we chose 300 μM PA to induce lipotoxic effects in HepG2 hepatocytes.

Several published studies investigated the hepatoprotective effects of propolis collected from different geographic origins (Banskota et al., 2000, 2001). Propolis extracts can protect against liver damage induced by various hepatotoxic regents, including paracetamol and carbon tetrachloride in animal studies (Madrigal-Santillan et al., 2014). *In vitro*, propolis reduced D-galactosamine/*tumour necrosis factor*-α (TNF-α)-induced cell death in primary cultured murine hepatocytes (Banskota et al., 2000, 2001). To investigate whether CP can decrease PA-induced HepG2 cell death, various concentrations of CP were used for pre-treatment 2 h before 300 μM PA administration. The decreased cell viability and LDH release induced by PA were rescued by CP pre-treatment in a dose–response manner. Using another human steatotic hepatocyte cell line, L02, we also reestablished our palmitate induced hepatocyte cellular damage model and similar protective effects were found (Fig. 1b and d). Moreover, the protective effects of CP could not be attributed to its toxic effects on the cells because 15 μg/ml of CP showed no change in the HepG2 and L02 cell viability.

3.3. Chinese propolis protects against palmitic acid induced mitochondrial ATP depletion and lipoapoptosis in HepG2 cells

The increased FFA metabolites will have direct detrimental effects on the mitochondria by decreasing ATP levels, leading to lipoapoptosis in the hepatocytes. We found that the intracellular ATP levels dramatically decreased after PA treatment, while CP attenuated PA's effects on ATP reduction (Fig. 2a). Furthermore, by determining the cellular caspase 3/7 levels, we found that the lipoapoptotic effects by PA were also rescued by CP pre-treatment. The mitochondrial damage induced by FFAs lead to a decrease in the $\Delta\Psi m$, which was followed by ATP depletion (Xiao, Waldrop, Khimji, & Kilic, 2012). Lipoapoptosis plays key roles in lipotoxic liver injury and NASH (Xiao et al., 2015). In parallel with previous studies, saturated FFAs (PA), but not unsaturated FFAs, stimulate lipoapoptosis in the hepatocytes by increasing the caspase 3/7 levels (Malhi, Bronk, Werneburg, & Gores, 2006). Our results provide additional evidence that CP contributes to restoring the energy provision and preventing lipoapoptosis in liver cells.

3.4. Chinese propolis ameliorated oxidative stress and inflammation induced by palmitic acid in HepG2 cells

Oxidative stress of the liver is considered a critical event in the "second-hit" during NAFLD pathological progression. Therefore, improving the cellular antioxidant potential is important for NAFLD treatment. We determined cellular oxidative stress parameters (total antioxidant capacity, T-AOC, and super oxide dismutase, SOD) as well as several important antioxidant/detoxificant gene expressions (*GSTA1*, *TXNRD1* and *NQO-1*) (Lennicke et al., 2017). PA leads to a loss of T-AOC, but it has limited effects on

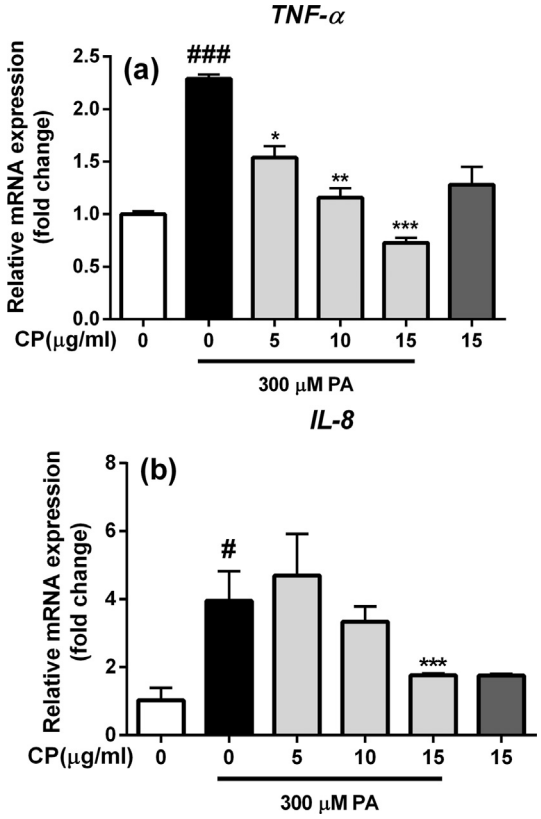

Fig. 4. Chinese propolis protects palmitic acid-induced pro-inflammatory cytokine expressions in HepG2 cells. HepG2 cells were pre-treated with various concentrations of CP followed by 12 h of PA (300 μM) treatment. The expression levels of two main pro-inflammatory cytokine genes, TNF-α (a) and IL-8 (a), are shown. Data are shown as the mean ± SD from three independent experiments. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ indicate significant differences compared to untreated cells. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate significant differences compared to PA-treated cells.

SOD as well as other antioxidant/detoxificant genes. Interestingly, CP led to a dramatic increase in these antioxidant parameters (Fig. 3). In experimental animal liver damage models, propolis administration can increase hepatic antioxidant enzyme activity, including SOD, catalase, and glutathione peroxidase (GPx) (Kismet et al., 2008; Nakamura, Osonoi, & Terauchi, 2010; Seo, Park, Song, Kim, & Yoon, 2003). Similar to our previous studies in murine macrophages (Wang et al., 2014, 2015), we found that CP has strong induction effects on cellular antioxidant genes. Nevertheless, chemical composition of propolis varies with their geographic origins, reflecting the diversity of local plants. The propolis sample used in the present study is collected from poplar trees (*Populus* sp.), which has different chemical characters compared with other propolis types. We recently conducted a comparative study using propolis extracts from China (derived from poplar) and Brazil (derived from *Baccharis dracunculifolia* DC). Intriguingly, these two types propolis shown some bioequivalence in different inflammatory models, by decreased serum proinflammatory cytokine concentrations in mice and inhibited inflammation transcription factor NF-κB activation (Wang et al., 2015). Therefore, we inferred that Brazilian green propolis might have a great therapeutical potential against palmitic acid induced hepatocytes damages.

Accompanying the oxidative stress, excessive fat accumulation in hepatocytes with overexpression in inflammatory cytokines can lead to neutrophil infiltration and trigger inflammatory injury (Guo et al., 2015). PA's inductive effects on the expressions of inflammatory cytokines TNF-α and IL-8 were significantly inhibited by CP (Fig. 4). In the patients diagnosed with NAFLD, the inflammatory transcription factors NF-κB and AP-1 can be activated by lipid peroxidation products. In our previous studies, bacteria endotoxin induced inflammation and the activation of NF-κB and AP-1 was inhibited by CP, which might be a reasonable explanation for its anti-inflammatory effects in liver cells (Wang et al., 2013, 2015). These data suggest that the potent antioxidant and anti-inflammatory effects of CP might act synergistically against lipoapoptosis in the liver.

3.5. Chinese propolis induced Nrf2 expression and activates HO-1, which acts against palmitic acid overload in HepG2 cells

The Nrf2 transcription factor is known as a master regulator in cellular defence responses through activating ARE phase II antioxidant enzymes, such as haeme oxygenase (HO)-1(Lever, Boddu, George, & Agarwal, 2016). HO-1 is known as a stimulation response protein that is induced in various stress conditions (Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016). Inductive production of HO-1 has beneficial effects in various pathological processes, such as oxidative stress, inflammation, ischaemia/reperfusion injury, and transplant rejection. This production's beneficial roles in the liver have been investigated in an experimental steatohepatitis model (Nan et al., 2010). We observed that CP increases the HO-1 gene expression in PA treated HepG2 cells in a dose-dependent manner (Fig. 5a). As HO-1 is one of the most rep-

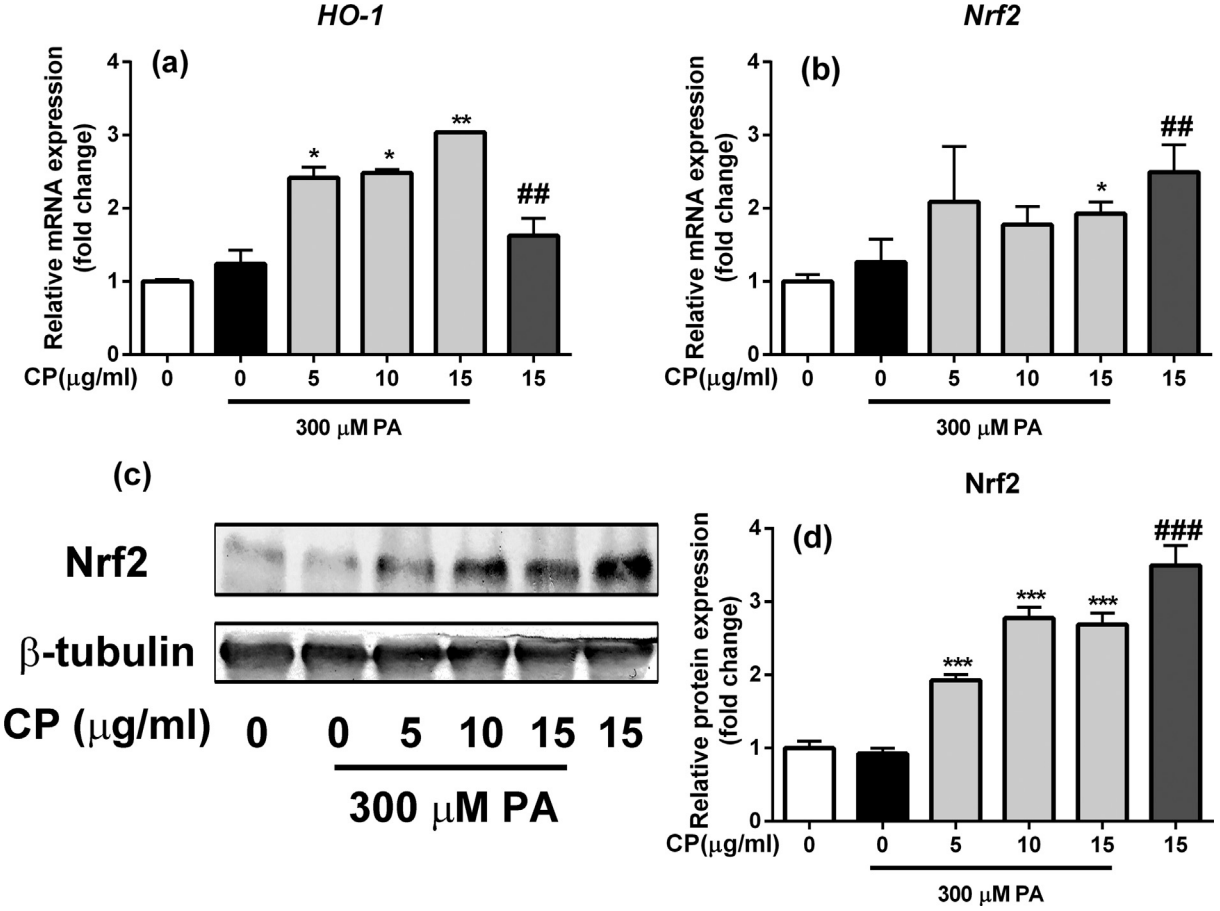

Fig. 5. Chinese propolis induced Nrf2 expression and activated HO-1, which worked against palmitic acid overload in HepG2 cells. HepG2 cells were pre-treated with various concentrations of CP followed by 12 h of PA (300 μM) treatment. HO-1 (a) and Nrf2 (b) gene expression levels are shown. $^{\#\#}p < 0.01$ indicates significant differences compared to untreated cells. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant differences compared to PA-treated cells. (c) Cytoplasmic Nrf2 protein was evaluated by Western blot and quantified data (d) are expressed in arbitrary units as the mean ± SD of three experiments. # indicates significantly different from untreated cells. $^{\#\#\#}p < 0.001$ indicates significant differences compared to untreated cells. $^{***}p < 0.001$ indicates significant differences compared to PA-treated cells.

resentative ARE response enzyme regulated by Nrf2, we tested the Nrf2 expression at both the gene and protein levels in PA treated hepatocytes (Fig. 5b–d). The promising inductive effects of CP on Nrf2 further provide mechanistic evidence for its hepatoprotective effects in this study. Our recent studies also identified several active molecules from CP which activate Nrf2-ARE signaling, including CAPE, kaempferol, quercetin, and pinocembrin (Wang et al., 2016). Further studies are required to evaluate the effects and mechanisms of these single molecules responsible for its anti-oxidative effects against PA-induced hepatocytes damages.

4. Conclusions

This study demonstrates that Chinese propolis protects hepatocytes against palmitic acid-induced lipotoxicity by lowering biomarkers of apoptosis, oxidative stress and inflammation. The hepatoprotective effects of Chinese propolis may involve the activation of Nrf2. These findings encourage the future use of CP for the prevention and/or treatment of NAFLD and NASH and their complications.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.04.039.

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