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Short communications

Protective effect and mechanism of action of mulberry marc anthocyanins on carbon tetrachloride-induced liver fibrosis in rats

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ABSTRACT

Mulberry fruit is widely consumed either fresh or in processed food products such as juice, mulberry jelly and jam. Conversely, mulberry marc, the solid component after juicing, is primarily a waste product or used in animal fodder, despite being rich in anthocyanins. Anthocyanins have been reported to have hepatoprotective, hypolipidaemic and antioxidant activities. In this study, the protective effect of mulberry marc anthocyanins (MMA) on carbon tetrachloride (CCl_4)-induced liver fibrosis in rats was investigated. Male Sprague Dawley rats were injected with CCl_4 to induce liver fibrosis. Compared with the model group, the contents of alanine aminotransferase, aspartate amino transferase, hyaluronidase acid, hydroxyproline and collagen type-III in the MMA 200 mg/kg group were decreased. Moreover, pathological examination and immunohistochemical showed that CCl_4-induced liver fibrosis was alleviated by administration of MMA. These results demonstrate that MMA has a potent protective effect on CCl_4-induced liver fibrosis in rats.

1. Introduction

Liver fibrosis is caused by excessive accumulation of extracellular matrix (ECM), especially collagen protein. It is a pathological process that often leads to hepatocellular carcinoma (Iwaisako, Brenner, & Kisseleva, 2012), which is one of the major causes of morbidity and mortality worldwide (Jia et al., 2015). Carbon tetrachloride (CCl_4) is commonly used to induce liver injury, and it has been shown that the CCl_4-induced liver oxidative damage responses in animals are similar to those in humans (Hsu et al., 2008). CCl_4 is metabolized by cytochrome P450 to trichloromethyl radical ($\cdot CCl_3$) and trichloromethyl peroxide ($\cdot OOCCl_3$) radical (Kang et al., 2008). These radicals initiate radical-mediated lipid peroxidation, prior to damaging the liver cell membrane and causing acute liver injury (Khan, Khan, Sahreen, & Shah, 2012). It has been indicated that products from natural and medicinal plants show high capacity for scavenging CCl_4-induced free radicals (Hsiao et al., 2003). However, the effect of these plants on certain

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Chemical compounds: Cyanidin-3-O-glucoside (PubChem CID: 441667); Delphinidin-3-O-glucoside (PubChem CID: 443650); Petunidin-3-O-glucoside (PubChem CID: 443651); Peonidin-3-O-glucoside (PubChem CID: 443654); Malvidin-3-O-glucoside (PubChem CID: 443652); Pelargonidin-3-O-glucoside (PubChem CID: 443648).

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disorders, including liver fibrosis has not been studied. Therefore, investigating possible natural products for protecting liver fibrosis is attracting increased interest in medical science.

Anthocyanins are natural and safe pigments, which are mainly found in coloured fruits and vegetables, such as eggplant, blueberry and mulberry (Yong, Jae, Jun, Young, & Hye, 2011). It has been reported that 6 common anthocyanins and their glycosides occur in natural world, such as cyanidin-3-O-glucoside, delphinidin-3-O-glucoside and petunidin-3-O-glucoside (Kalisz, Kalisz, & Oszmian'ski, 2004). Anthocyanins comprise a series of low molecular weight flavonoid compounds. It have been shown many biological activities, such as anticancer, hepatoprotective (Hou, Zhang, Zhang, Su, & Wei, 2013). Jiang, Tang, Zhang, Liu, and Guo (2014) reported that cyanidin-3-O-β-glucoside protects primary mouse hepatocytes against high glucose-induced apoptosis by modulating mitochondrial dysfunction and the PI3K/Akt pathway. Sun et al. (2012) reported anti-tumour activity of cyanidin-3-O-glucoside from Chinese bayberry fruit. In addition, Antonio et al. (2015) reported delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside mediate the redox-sensitive caspase 3-related pro-apoptotic effect of blackcurrant juice on leukaemia Jurkat cells. Mulberry is in the genus *Morus* and belongs to the family of *Moraceae*. Mulberry fruit is rich in anthocyanins, and in China it is often consumed as juice because of difficulties in storage of the fruit (Suh, Noh, Kang, Kim, & Lee, 2003). However, mulberry marc, the solid component after juicing, is primarily a waste product or used in animal fodder. We have previously found the content of anthocyanins to be higher in mulberry marc compared with mulberry juice, with the major composition of mulberry marc anthocyanins (MMA) being cyanidin-3-glucoside (cy-3-glu). Moreover, we have shown that MMA markedly reduces fatigue in a murine exhaustion model (Jiang et al., 2013). In the current study, therefore, the protective effect of MMA following CCl_4-induced liver fibrosis in rats was investigated. Furthermore, we explored the possible mechanism of the protective effect of MMA on CCl_4-induced liver fibrosis in rats. These results provide a basis for investigation of prevention of liver fibrosis by MMA.

2. Materials and methods

2.1. *High performance liquid chromatography (HPLC) determination of MMA*

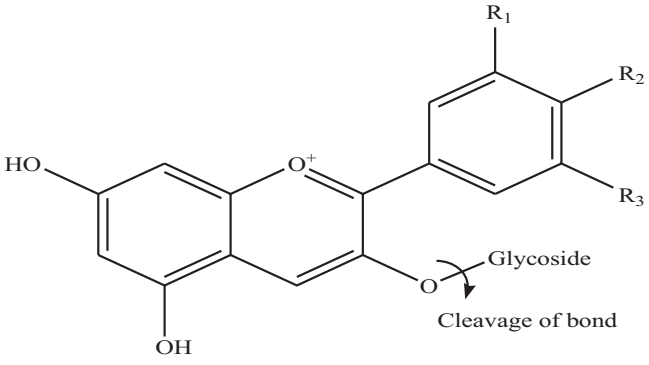
Purified mulberry marc products (0.01 g) and cy-3-glu (0.0018 mg) standard were dissolved in methanol solution 2% hydrochloric acid (pH 3) to a volume of 10 mL, prior to passing through a 0.45-μm membrane filter, to obtain sample and standard solutions. These solutions were analysed using a Waters 2695 HPLC with a Waters 2996 PDA diode array detector with the following chromatographic conditions: SunfireC18 column (5 μm, 4.6 mm × 250 mm). Column temperature was set at 20 °C and at a flow rate of 1.0 mL/min. The mobile phase included the use of acidified 2% methanol as solvent A, and water/methanol/acetonitrile/acetic acid at a ratio of 160:90:90:40 (v/v/v/v) as solvent B. The gradient programmes were as follows: at 0–1 min, 100% A; 1–3 min, 95% A; 3–6 min, 93% A; 6–30 min, 75% A; 30–40 min, 35% A; 40–46 min, 0% A; 46–50 min, 93% A. Anthocyanins were determined at 530 nm. The cy-3-glu standard was used to identify unknown sample peaks. The content measurements of each sample were repeated three times.

2.2. *Experiment design*

Male Sprague Dawley (SD) rats weighing 180–200 g were provided by the Zhejiang Academy of Medical Science, Zhejiang China (No. SC 2008–3344). All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of Zhejiang and were approved by the Committee on the Ethics of Animal Experiments at Zhejiang Agriculture and Forestry University. SD rats (n = 60) were divided into six groups: Group 1 rats were injected with peanut oil twice per week for 6 weeks and orally administered distilled water daily for 2 weeks (control group); Group 2 rats were injected with CCl_4 (20% in peanut oil) twice per week for 6 weeks and administered distilled water for 2 weeks (model group); Group 3 rats were injected with CCl_4 (20% in peanut oil) twice per week for 6 weeks and administered colchicine (0.2 mg/kg) for 2 weeks (colchicine group); Groups 4, 5 and 6 rats were injected with CCl_4 (20% in peanut oil) twice per week for 6 weeks and administered MMA (200, 400 or 800 mg/kg body weight, respectively) daily for 2 weeks. Blood serum was stored at –80 °C for biochemical assays (serum levels of ALT, AST, HA, HYP and Col III were examined using a microplate reader). Rat livers and spleens were collected and weighed.

2.3. *Histopathological evaluation and immunohistochemical staining*

Sections (4 μm) of liver tissue were stained with Masson's trichrome and observed using a light microscope (BX20, Olympus, Tokyo, Japan). Sections were then counterstained with haematoxylin, dehydrated using graded alcohol and xylene, and mounted with Entelan. Immunostaining intensities were analysed by microscopy (BX20, Olympus).



Pelargonidin	R_1=H; R_2=OH; R_3=H
Cyanidin	R_1=OH; R_2=OH; R_3=H
Peonidin	R_1=OMe; R_2=OH; R_3=H
Delphinidin	R_1=OH; R_2=OH; R_3=OH
Petunidin	R_1=OMe; R_2=OH; R_3=OH
Malvidin	R_1=OMe; R_2=OH; R_3=OMe

Fig. 1 – Structure of 6 common anthocyanins.