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Understanding the effect of anthocyanins extracted from purple sweet potatoes on alcohol-induced liver injury in mice

Zhan Cai[a], Lihua Song[a], Bingjun Qian[c], Wei Xu[a], Junkun Ren[a], Pu Jing[a,*], Indrawati Oey[b,*]

[a] Research Center for Food Safety and Nutrition, Key Lab of Urban Agriculture (South), Bor S. Luh Food Safety Research Center, School of Agriculture & Biology, Shanghai Jiao Tong University, Shanghai 200240, China

[b] Department of Food Science, University of Otago, PO BOX 56, Dunedin 9054, New Zealand

[c] Department of Preventive Medicine, Jiangsu Vocational College of Medicine, Jiangsu 224005, China

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ABSTRACT

Anthocyanins as antioxidants are potential to protect liver from alcoholic damage, but might be pro-oxidants under certain conditions. In this study, twelve purple sweet potatoes anthocyanins (PSPA) were isolated and their effects on alcohol-induced liver injury were studied. These PSPA were rich in cyanidin derivatives and fed to male C57BL/6 mice as colorants in alcoholic drink with low, median, or high dosages PSPA *i.e.* 50, 100, or 300 mg/kg·BW·d. Compared to the alcohol group, the median-dose PSPA showed a clear improvement in the liver indexes/histology, serum ALT level, oxidative stress status, and even a recovery to the normal level, however the high dose PSPA promoted the liver injury *via* a pro-oxidant effect, as reflected by increased malondialdehyde (MDA) content and decreased GSH level. The results suggested that cyanidin derivatives with an *ortho*-hydroxyl structure on B-ring might promote the oxidative stress of alcohol-induced liver injury at high doses as a pro-oxidant.

1. Introduction

Alcoholic beverages have been part of the human diet long before recorded history. Consumption of excessive alcohol might seriously damage human health, such as alcoholic liver disease (ALD), which is one of the major causes of morbidity and mortality worldwide (Smyth et al., 2015;). The pathogenesis of ALD involves complex and still unclear biological processes. The oxidative metabolites of ethanol such as acetaldehyde and reactive oxygen species (ROS) play a preeminent role in the clinical and pathological spectrum of ALD. Acetaldehyde induces Kupffer cells to release ROS and proinflammatory cytokines and chemokines that contribute to neutrophils infiltration. ROS was massively produced by excessive alcohol consumption through the induction of microsomal protein cytochrome P450 2E1 (CYP2E1), contributing to the alcohol oxidation to acetaldehyde, mainly at increasing alcohol concentrations as well as subsequent their induction by chronic alcohol abuse (Azuma, Kumazawa, Miya, Mabuchi, & Nishida, 2008). Additionally, overgeneration of ROS inflicts cell death and tissue damage by targeting vital cellular components such as DNA, lipids, and proteins, thus arising as key players in disease pathogenesis (Mari et al., 2010; Molina, Gardner, Souza-Smith, & Whitaker, 2014).

Numerous antioxidants have exhibited a protection against alcoholic liver damage as reported in previous studies (Singal, Jampana, & Weinman, 2011). Natural phytochemicals play important roles in the prevention of oxidative stress-related diseases (Aiyer, Warri, Woode, Hilakivi-Clarke, & Clarke, 2012). Among those, anthocyanins that are responsible for the attractive bright red, purple, violet, and blue colors in fruits, vegetables, and cereals (Chen, Xiang, Deng, Liu, & Li, 2013) have been reported to provide health benefits including antioxidation (Aqil et al., 2012), anti-inflammation (Jean-Gilles et al., 2012) and anti-cancer (Wang & Stoner, 2008). The consumption of anthocyanins has been considered to be associated with a reduced risk of developing chronic diseases (Del Rio et al., 2013). Additionally, it has been reported that anthocyanins can decrease hepatic lipid accumulation and counteract oxidative stress and hepatic inflammation (Jiang et al., 2016; Valenti et al., 2013). Therefore, anthocyanins are expected to be promising dietary antioxidants for preventing or alleviating ALD. However recent studies have reported that phenolic compounds such as anthocyanins have been proven to exert prooxidant activity under certain conditions, including high pH, high phenolic concentrations and the presence of redox-active transition metals (Eghbaliferiz & Iranshahi, 2016; Prochazkova, Bousova, & Wilhelmova, 2011).

Therefore, the aim of this research was to understand the effects of

* Corresponding authors.
E-mail addresses: pjing@sjtu.edu.cn (P. Jing), indrawati.oey@otago.ac.nz (I. Oey).

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supplementation of liquor colored with various doses of anthocyanins on alcoholic liver injury. Anthocyanins were extracted and isolated from purple sweet potatoes (PSP) *via* an aqueous two-phase extraction. The extraction condition was optimized using Box-Behnken design. The isolated anthocyanins were added in an alcohol drink containing 52% (v/v) ethanol. The colored alcohol drinks were fed to mice on daily basis for 6-week duration, and then the main liver function indexes, hepatic histological change and their oxidative status were investigated. To understand the antioxidative and prooxidative effects of anthocyanins on alcohol-induced liver injury, enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GP_X) and catalase (CAT); non-enzymatic antioxidant such as glutathione (GSH) and oxidative damage marker such as lipid oxidation were used in this study.

2. Materials and methods

2.1. Materials and reagents

Purple sweet potatoes (*Ipomoea batatas cv.* Ningzi 4) were purchased from local market in Shanghai, China. Commercial assay kits for analysis of glutathione peroxidase (GPx, A005), total superoxide dismutase (*T*-SOD, A001-1), catalase (CAT, A007-1), glutathione (GSH, A006), malondialdehyde (MDA, A003-1) and protein content (Bradford, W042) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All LC-MS grade solvents and other chemicals (analytical grade) were from Sigma (Shanghai, China). Ultrapure water prepared by a Leader-A1 Water Purification System with < 10 μg/L TOC and resistivity of 18.2 mΩ (Leader Instrument, Shanghai, China) was used for all experiments.

2.2. Extraction and purification of purple sweet potato anthocyanins (PSPA)

Purple sweet potatoes (PSP) were washed, peeled, diced and dried in a stove at 50°C for 4 hours. The dry potato pieces were ground in a JYZ-D57 food processor (Joyoung, China) to a particle size that could pass through a 40-mesh screen (pore size in diameter: 425 μm). The resulting powder fraction after passing the sieve was later referred as "extract powder". The extract powder was packed in sealed polyethylene plastic bags and afterwards stored in a refrigerator at 7 °C.

Ammonium sulfate/ethanol two-phase system with a solid-liquid ratio of 1:5 (w/w) between the extract powder and the extraction solvent was used to isolate anthocyanins. In this research, the Box-Behnken design was applied to determine effects of extraction variables and their main effects. Three-level factors were selected based on results of their single factorial assays and listed in Table S1 (supplementary materials). The pH (X_1) of the mixture was adjusted with hydrochloric acid (5 M) to 3.00, 3.50 and 4.00. Ethanol (X_2) was added to reach a mass fraction of 20, 22.5 and 25% (w/w). Then ammonium sulfate (X_3) was added to reach a mass fraction of 24, 25 and 26% (w/w). The response functions (Y) were anthocyanins yield (Y_1), purity (Y_2), and partition coefficient of anthocyanins (Y_3). Anthocyanin yield (Y_1) was expressed as the ratio of anthocyanins (mg) partitioned in ethanol phase (the top phase) to PSP powder (g). Anthocyanin purity (Y_2) was expressed as the ratio of anthocyanins (g) to the total extract powder (g). Partition coefficient of anthocyanins (Y_3) is the ratio of anthocyanin concentrations in the top (ethanol phase) and in the bottom (ammonium sulfate) phases. A set of 17 experiments was carried out with five replicates at the center (0, 0, 0) of the design (Table S2, supplementary materials). Each experiment was performed in triplicate and an average of the results was calculated.

The ammonium sulfate (X_3)/ethanol (X_2) two-phase system was added to the pre-weighted extract powder with a fixed solid-liquid ratio of 1:5 (w/w) and the pH (X_1) was adjusted based on the set of 17 experiments. All mixtures were then mixed thoroughly using a magnetic stirrer for 30 minutes at 20 °C and afterwards transferred into a separating funnel for 4–5 h to allow phase separation. The top and bottom phases of the system were collected and further analyzed for anthocyanins content as well as the total mass.

Numerical method based on desirability function was conducted to optimize the three responses (Myers & Montgomery, 2002). The desirability function reflects the desirable ranges for each response (d_i) in Eq. (1) ranging from 0 to 1 with (d_i) = 0 representing a completely undesirable and (d_i) = 1 representing a completely desirable or ideal response value.

$$D = (d_1 \times d_2 \times \ldots \times d_k)^{\frac{1}{k}} = (\Pi_{i=1}^{k} d_i)^{\frac{1}{k}} \quad (1)$$

where k is the number of responses in the measurement and d_i is the desirable range for each response. All the desired responses of anthocyanin yield, purity, and partition coefficient were the maximum of desirability function.

The anthocyanin-rich extract from PSPs was further purified using a 600 cm × 50 cm Amberlite XAD-7HP column (Huideyi, Beijing, China). Anthocyanin-rich extract dissolved in distilled water and applied to the column, followed by two columns of distilled water containing 0.01% HCl to remove water-soluble compounds. The anthocyanin fraction was eluted with ethanol at a flow rate of 1 mL/min and collected. The ethanol was removed from anthocyanin fractions under vacuum conditions at 40 °C.

The PSP anthocyanins were further purified in an LC-3000 preparative HPLC system (Chuangxin Tongheng Science and Technology Co. Ltd, Beijing, China). The preparative HPLC system was equipped with an UV/VIS detector and a Daisogel C_{18} column (30 mm × 250 mm, 9 μm, Daiso, Osaka, Japan). The solvent system consisted of ultra-pure water containing 1% (v/v) acetic acid as fluid phase A and methanol containing 1% (v/v) acetic acid as fluid phase B. A gradient program was used as follows: 0–3 min 30% B; 3–10 min 50% B; 10–12 min 70% B. The flow rate was 20 mL/min while the detection wavelength was 520 nm. The injection volume was 5 mL. Anthocyanin fractions were collected at different elution times. The methanol and some acetic acid in the fractions were removed by using rotary evaporators at 45 °C (Rotovapor, Yarong, China) up to about 20 mL. The residue was then frozen at −78 °C and lyophilized in a freeze-dry system (FD-1A-50, Beijing Boyikang, China). The dried anthocyanin isolates were stored in a refrigerator at 4 °C until further analysis.

2.3. Quantification of total monomeric anthocyanins

The concentration of total monomeric anthocyanins were determined by using an L5S UV–visible spectrophotometer (Shanghai Analytical Instrument, China) based on the pH differential method (Giusti & Wrolstad, 2001). Cuvettes with 1-cm path length were used. The absorbance values of the samples at 520 nm and 700 nm were recorded. The anthocyanin content was expressed as cyanidin-3-glucoside (MW: 449.2) equivalents, using the molar absorptivity of 26,900 L cm^{-1} mg^{-1}. The measurements were independently performed in triplicates.

2.4. Identification of anthocyanin fractions

The determination of high-resolution masses of anthocyanins in the fractions obtained earlier was carried out by using a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) coupled with the Waters Synapt HDMS Q-TOF MS detector. The analytical column was COSMOSIL C18-MS-II (3.0 × 150 mm, 5 μm, Nacalai Tesque, Tokyo, Japan). The freeze-dried anthocyanin fraction was dissolved in menthol and filtered through 0.22 μm nylon membrane filters (Shanghai Mosu Scientific Instruments and Materials, China). The injection volume was 5 μL and the autosampler temperature was set at 4 °C. The mobile phase consisted of solvents A (formic acid: water = 1:100, v/v) and B (formic

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