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Identification of the free phenolic profile of Adlay bran by UPLC-QTOF-MS/MS and inhibitory mechanisms of phenolic acids against xanthine oxidase

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ABSTRACT

Adlay bran free phenolic extract has been previously demonstrated to possess potent xanthine oxidase (XOD) inhibitory activity. The aims of this study were to characterize the free phenolic profile of adlay bran and investigate the structure-activity relationship, underlying mechanism and interaction of phenolic acids as XOD inhibitors. A total of twenty phenolics including ten phenolic acids, two coumarins, two phenolic aldedhyes and six flavonoids were identified in a phenolic compound-guided separation by UPLC-QTOF-MS/MS. Adlay bran free phenolic extract possessed strong XOD inhibitory activity related to hydroxycinnamic acids with methoxyl groups. The hydrogen bonding and hydrophobic interactions were the main forces in the binding of adlay phenolics to XOD. Sinapic acid, identified in adlay bran for the first time, possessed strong XOD inhibitory activity in a mixed non-competitive manner, and synergistic effects with other adlay phenolic acids at low concentrations, and would be a promising agent for preventing and treating hyperuricemia.

1. Introduction

Hyperuricemia is mostly due to regular intake of high purine-containing foods and invariably accompanied with gout and chronic kidney disease (Becker et al., 2005). Xanthine oxidase (XOD), an important enzyme associated with hyperuricemia, catalyzes the oxidation of xanthine to uric acid, causing the generation of reactive oxygen species (Chu, Chen, Wu, & Hsieh, 2014). Allopurinol, an effective XOD inhibitor, has been clinically used for the treatment of hyperuricemia and gout (Wang, Zhang, Pan, & Gong, 2015). However, allopurinol causes hepatitis, nephropathy, hypersensitivity and skin rash, raising safety concerns (Li, Zhang, Wang, Xie, & Kong, 2011). The alternative XOD inhibitors with minimal side effects for preventing and treating hyperuricemia are highly desirable.

Adlay (*Coix lachryma-jobi L. var. ma-yuen* Stapf), a nourishing soft-shelled seed crop widely distributed in China, Japan and India, is used as a dietary supplement for humans and traditional Chinese medicine due to the bioactive phytochemicals including phenolic acids, phenolic aldehydes, phenolic alcohols, flavonoids, lignans, steroids and lactams (Chung et al., 2011; Wu, Charles, & Huang, 2007). The previous studies indicated that adlay bran exhibited more effective anti-haemolysis (Zhao, Yang, Lin, Sun, & Wang, 2017), XOD inhibitory (Zhao et al., 2014) and anti-proliferative activities (Chung et al., 2011) than endosperm. Phenolics are the major phytochemicals in adlay bran and contributed largely to antioxidant (Yang, Zhao, & Lin, 2016), anti-haemolysis (Zhao et al., 2017), XOD inhibitory (Zhao et al., 2014), anti-allergic (Chen, Lo, & Chiang, 2012), anti-inflammatory (Chen, Chung, Chiang, & Lin, 2011) and gastroprotective activities (Chung et al., 2011).

Previous work from our group has shown that brown adlay extract could effectively decrease the serum uric acid levels of oxonate-induced hyperuricemic rats without any side effects (Zhao et al., 2014). The adlay bran free phenolic extract exhibited stronger XOD inhibitory, antioxidant and anti-haemolysis activities than the adlay bran bound phenolic extract (Zhao et al., 2017, 2014). The most common phenolic compounds found in whole grains are phenolic acids and flavonoids. However, the content and type of phenolics varied depending on the type of cereal and variety (Liu, 2007). Identification of the free phenolic profile of adlay bran is needed to illustrate the relationship between the phenolics and pharmaceutical activity of adlay bran.

Flavonoids widely distributed in fruits and vegetables have attracted growing attention. The structure-activity relationship of dietary flavonoids as XOD inhibitors is clear. Chrysin, apigenin, luteolin, kaempferol and quercetin were considered as the effective XOD inhibitors (Cos et al., 1998; Dong et al., 2016; Nagao, Seki, & Kobayashi, 1999). Nevertheless, the structure-XOD inhibitory activity relationship

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of phenolic acids is not well understood due to different test methods (Masuda et al., 2014; Nile & Park, 2014; Ozyurek, Bektasoglu, Guclu, & Apak, 2009; Valentao et al., 2001). It is of high interest to evaluate the relationship between the chemical structures of phenolic acids and their XOD inhibitory activities and verify the potential use of phenolic acids as XOD inhibitors. In addition, the combination of phenolic acids is potentially useful for preventing and treating hyperuricemia.

In the current study, the free phenolic profile of adlay bran was characterized by UPLC-QTOF-MS/MS. The structure-activity relationship of phenolic acids found in adlay bran as XOD inhibitors was illustrated. The underlying mechanisms of their XOD inhibitory effects were also investigated. The interactions of adlay phenolics on XOD inhibition activity were conducted. This study is expected to benefit further application of adlay phenolic acids in functional foods for preventing and treating hyperuricemia.

2. Materials and methods

2.1. Chemicals and materials

XOD (7.2 units ml^{-1}, from bovine milk), xanthine, allopurinol (> 98%), sinapic, ferulic, syringic, *p*-coumaric, chlorogenic and caffeic acids (> 98%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the solvents for HPLC analyse were of HPLC grade. Methanol for UPLC analyse was of LC-MS ultra-quality (VWR International, Leuven, Belgium). Deionized water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Water containing 0.1% formic acid was of LC-MS ultra-quality (Sigma, St. Louis, MO, USA). All other chemicals were of analytical grade. XAD-16 resin was purchased from H&E Co., Ltd., Beijing, China. Air-dried adlay bran was purchased from Shenlong adlay cooperative (Fujian, China) in August 2016, ground into fine powder and stored at 4 °C until use.

2.2. Characterization of free phenolic profile of adlay bran by UPLC-QTOF-MS/MS

Adlay bran free phenolics were extracted and enriched according to our previously published method (Zhao et al., 2014; Yang et al., 2016). The elution fractions (30%, 50% and 70% ethanol fractions) containing about 95% of adlay bran free phenolics were further purified by solid-phase extraction (SPE) cartridges (C18, 50 μm, 500 mg, 3 ml, Supelco, Bellefonte, USA). The elution fractions possessing high phenolic contents were filtered through 0.22 μm of nylon membrane.

UPLC-QTOF-MS/MS was performed on a Waters Acquity UPLC I Class (Waters, Hertfordshire, UK) equipped with an Impact II ESI-QTOF-MS (Bruker Daltronics, Bremen, Germany). Mass spectrometric analysis was performed in negative ion mode in the mass range of 60–1000 Da with the following mass spectrometer settings: capillary = 3000 V; nebulizer gas = 2 bar; drying gas = 6 L/min; drying temperature = 180 °C. The acquisition of fragment spectra for each mass was carried out using MS/MS mode with collision energy set to 40 eV. Nitrogen was used as nebulising, drying and collision gas. The analyte was separated on a Waters Acquity CORTECS C18 column (2.1 mm × 100 mm, 1.6 μm) at 30 °C with a flow rate of 200 μl/min. The solvent system consisted of (A) 0.1% (v/v) formic acid and (B) methanol. The gradient conditions were: 90% A in 0–2 min; 90–0% A in 2–10 min; 0–90% A in 10–11 min; 90% A in 11–12 min. The injection volume was set to 1 μl. Identification of free phenolics was carried out using the exact mass, isotope ratio and comparison of detected fragment ions with online databases including PubChem and Chemspider for prediction of chemical and structural formulas.

2.3. Determination of XOD inhibitory activity

XOD activity was determined by measuring the formation of uric acid from xanthine according to our previously published method (Zhao et al., 2014). All solutions were prepared in 50 mM phosphate buffer (pH 7.4). Fifty microliters of a test sample and 50 μl of XOD (0.1 U/ml) were added in a well of a 96-well microplate and pre-incubated at 25 °C for 5 min before 150 μl of 0.42 mM xanthine was added. The resultant mixture was incubated at 25 °C for 30 min. The enzymatic reaction was stopped by adding 80 μl of 1 M HCl. The reaction mixture was diluted ten times for HPLC analysis. HPLC analysis was carried out using a Waters e2695 HPLC system (Waters, Milford, MA, USA) equipped with a Waters 2998 photodiode array detector (PDA). An Agilent XDB-C18 column (250 × 4.6 mm i.d., 5 μm, Santa Clara, CA, USA) and an isocratic mobile phase (i.e. 95% A in 0–10 min) consisting of (A) 10 mM NaH_2PO_4-Na_2HPO_4 buffer (pH 5.5) and (B) methanol were used. The flow rate was set at 1.0 ml/min. The detection wavelength was 290 nm. Phosphate buffer (50 mM, pH 7.4) was used as a negative control and allopurinol was used as a positive control.

$$\text{Relative enzymatic activity}(\%) = \text{peak area of uric acid of sample reaction} / \text{peak area of uric acid of negative control reaction} \times 100 \quad (1)$$

IC_{50} value (50% inhibitory concentration) was calculated by linear regression analysis. The plots of velocity (v, the production of uric acid per min) versus [XOD] at different concentrations of adlay phenolics were constructed using the same method.

2.4. Superoxide radical scavenging activity assay

The superoxide radicals $O_2^{-}\cdot$ were generated using the methods described previously (Wu et al., 2010). All solutions were prepared in 50 mM phosphate buffer (pH 7.4). Twenty microliters of a test sample, 180 μl of 0.6 mM NBT (nitro blue tetrazolium) and 20 μl of 3 mM xanthine were mixed in a well of a 96-well microplate, to which 20 μl of XOD (0.1 U/ml) was added to initiate the reaction. The mixture was incubated at 25 °C for 30 min and measured at 560 nm. Phosphate buffer (50 mM, pH 7.4) was used as a negative control and Trolox was used as a positive control.

$$\text{Superoxide radical scavenging activity}(\%) = [(\text{Absorbance}_{\text{negative control}} - \text{Absorbance}_{\text{sample}})/\text{Absorbance}_{\text{negative control}}] \times 100 \quad (2)$$

IC_{50} value (50% superoxide radical scavenging concentration) was calculated by linear regression analysis.

2.5. Lineweaver-Burk and Dixon plots

Lineweaver-Burk plots analysis was performed to determine the XOD inhibitory modes of sinapic, ferulic, syringic, *p*-coumaric, chlorogenic and caffeic acids. This kinetics study was conducted in the absence and presence of adlay phenolics with varying concentrations of xanthine as the substrate. The competitive inhibition constant (Ki) and non-competitive inhibition constant (αKi) were determined by Dixon plot analysis (Cornish-Bowden, 1974).

2.6. The effects of adlay phenolics on intrinsic fluorescence of XOD

Fluorescence quenching was determined according to Wang et al. (2015). Two milliliters of XOD (0.1 U/ml) was titrated by successive additions of 5 mM adlay phenolics to obtain the final concentrations of 0, 2.50, 5.00, 7.49, 9.98, 12.47, 14.96, 17.44 and 24.88 μM, respectively. The sample solutions were thoroughly mixed and equilibrated for 5 min at 298 K. Emission spectra were recorded from 290 to 500 nm at an excitation wavelength of 280 nm. Both excitation and emission slit widths were set at 2.5 nm. Fluorescence quenching is described according to the modified Stern–Volmer equation as follows:

$$F_0/(F_0 - F) = 1/f + 1/(f \times K_{sv} \times [Q]) \quad (3)$$