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Identification of a flavonoid *C*-glycoside as potent antioxidant

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ABSTRACT

Flavonoids have been documented to have good antioxidant activities *in vitro*. However, reports on the cellular antioxidant activities of flavonoid *C*-glycosides are very limited. In this work, an apigenin *C*-glycoside was purified from *Artocarpus heterophyllus* by column chromatography and was identified to be 2″-*O*-*β*-D-xylosylvitexin by nuclear magnetic resonance spectroscopy. The cellular antioxidant activity and anticancer activity of 2″-*O*-*β*-D-xylosylvitexin were evaluated for the first time. The quantitative structure-activity relationship was analysed by molecular modeling. Apigenin presented an unexpected cellular antioxidation behaviour. It had an antioxidant activity at low concentration and a prooxidant activity at high concentration, whereas 2″-*O*-*β*-D-xylosylvitexin showed a dose-dependent cellular antioxidant activity. It indicated that C-glycosidation improved the cellular antioxidation performance of apigenin and eliminated the prooxidant effect. The *ortho*-dihydroxyl at *C*-3′/*C*-4′ and *C*-3 hydroxyl in the flavonoid skeleton play important roles in the antioxidation behaviour. The cell proliferation assay revealed a low cytotoxicity of 2″-*O*-*β*-D-xylosylvitexin.

1. Introduction

Flavonoids are an important class of secondary metabolites, which have been generally recognized as beneficial to human body, and afford positive effects to health maintenance and disease prevention [1]. Plant flavonoids exhibit a wide spectrum of biological activities, such as anti-inflammatory and anticancer activities [2,3]. They also show good potential in prevention of cardiovascular diseases [4]. Therefore, some flavonoids have been applied in development of nutraceuticals and medicines.

Artocarpus heterophyllus is a fruit plant belonging to *Moraceae* family. The fruit is called jackfruit. It tastes sweet and comprises of abundant nutrients [5], which make it widely accepted by people in tropical and subtropical regions. The bark, leaf and wood have been reported with pharmaceutical activities, including anticancer, anti-inflammatory, hypoglycemic and wound healing effects [6]. These pharmaceutical activities indicate that *A. heterophyllus* is a good source of bioactive chemicals. This plant has been found to have abundant flavones, and prenylated flavones are the characteristic secondary metabolites [7]. Even though some of the phytochemicals in *A. heterophyllus* have been identified [6], there are lots of secondary metabolites remained unknown. To extensively understand the chemical composition of *A. heterophyllus* and possible application in medicines, it is worthy to purify and identify more phytochemicals from *A. heterophyllus*.

Increased intracellular oxidation stress will lead to damage to biomolecules like DNA, lipids and proteins. It is a main contributor to chronic diseases [8]. Plant flavonoids are well known for their antioxidant activities. However, previous antioxidant activity studies about flavonoids were mainly focused on the non-cellular antioxidant activity, and information on their antioxidant performance within cells is limited. Cellular antioxidant activity assay can quantify the antioxidant capacity of a bioactive compound within cells. Comparing to other chemical antioxidant protocols, this assay provides a comprehensive understanding of how antioxidants behave under physiological conditions. The uptake, distribution and metabolism of the antioxidants are taken into account. Flavonoids in *A. heterophyllus* is a promising resource of antioxidants. It is worthy to reveal the cellular antioxidant capacity for exploration of potent cellular antioxidant.

In the present work, a leading flavonoid was isolated from *A. heterophyllus* leaf and identified. The antioxidant activities were determined by oxygen radical absorption capacity (ORAC) assay, DPPH and hydroxyl radicals scavenging activity assays, and cellular antioxidant activity assay. Moreover, cytotoxicity and cancer cell

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proliferation inhibition activity of this compound against human $HepG_2$ hepatoma cell line and human MCF-7 breast carcinoma cell line were measured.

2. Materials and methods

2.1. Chemicals and analysis techniques

Trolox, 2,2′-azobis-amidinopropane (ABAP), 2,7-diacetate dichlorofluorescin, fluorescein sodium salt, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pyrogallol red (PGR), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 1,10-Phenanthroline, and fetal bovine serum were purchased from Sigma-AldrichCo. (St. Louis, MO, USA). Williams' medium E, Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), and other cell culture reagents were purchased from CASMART, China. All the other reagents used were of analytical grade.

Nuclear magnetic resonance (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz) spectra were recorded on a Bruker DRX-500 instrument (Bruker, Rheinstetten, Germany). CD_3OD (δ_H 3.31and δ_C 49.15 ppm) was used as reference. ^{1}H, ^{13}C, COSY, HSQC and HMBC spectra were recorded. Mass spectra were acquired on a MDS SCIEX API 2000 LC/MS apparatus (MDS Sciex, Ontario, Canada). Ultrahigh performance liquid chromatography was carried out with an Agilent 1260 Infinity liquid chromatograph (Agilent Technologies, CA, USA) equipped with a diode array detector and a ZORBAX Eclipse Plus C18 column (4.6 × 100 mm, 1.8 μm of particle size).

2.2. Sample preparation

Fresh *A. heterophyllus* leaves were collected from South China Botanical Garden, Chinese Academy of Sciences, China. They were carefully washed with distilled water, then dried in an oven at 50 °C for 24 h. The dried leaves were pulverized into powder by a laboratory mill (FW100, Taisite Instrument Co., Ltd, Tianjin, China).

2.3. Extraction, purification and identification

Dry leaf powders (1.0 kg) were extracted with 80% ethanol (10 L) at room temperature for one week. The extracts were collected and concentrated to give dark green solids. The extract was resuspended in deionized water and fractionated gradually by petroleum ether, chloroform and ethyl acetate. The ethyl acetate extract was concentrated to dryness at a low pressure. Three grams of ethyl acetate extract were subjected to purification by C18 column (15 × 460 mm, 50 μm of particle size) eluted gradually with methanol-H_2O. The methanol-H_2O (25:75) eluates were submitted to purification by semi-preparative HPLC equipped with a C18 column (10 × 250 mm, 5 μm of particle size, 120 Å of pore size) to obtain compound **1** (45 mg).

The NMR data of compound **1** are showed as below:

^{1}H NMR (CD_3OD, 500 MHz) δ: 6.61 (1H, s, H-3), 6.24 (1H, s, H-6), 7.98 (2H, d, J = 7.5 Hz, H-2′, H-6′), 6.94 (2H, d, J = 7.5 Hz, H-3′, H-5′); Glucose moiety (5.03 (1H, d, J = 10.0 Hz, H-1), 4.31 (1H, dd, J = 10.0, 7.5 Hz, H-2), 3.71 (1H, d, J = 7.5 Hz, H-3), 3.68 (1H, overlapped, H-4), 3.47 (1H, m, H-5), 3.81 (1H, overlapped, H-6a), 3.96 (1H, d, J = 12.5 Hz, H-6b)); Xylose moiety (4.13 (1H, d, J = 7.5 Hz, H-1), 2.97 (1H, t, J = 7.5 Hz, H-2), 3.08 (1H, t, J = 7.5 Hz, H-3), 3.15 (1H, overlapped, H-4), 2.59 (1H, dd, J = 10.0, 11.0 Hz, H-5a), 3.20 (1H, overlapped, H-5b)).

^{13}C NMR (CD_3OD, 125 MHz) δ: 166.6 (C-2), 103.7 (C-3), 184.3 (C-4), 105.7 (C-4a), 162.7 (C-5), 99.4 (C-6), 164.7 (C-7), 105.1 (C-8), 158.8 (C-8a), 123.7 (C-1′), 130.2 (C-2′, C-6′), 117.2 (C-3′, C-5′), 162.9 (C-4′). Glucose moeity (73.8 (C-1), 81.1 (C-2), 80.2 (C-3), 72.1 (C-4), 82.9 (C-5), 63.0 (C-6)). Xylose moeity (106.3 (C-1), 75.4 (C-2), 77.4 (C-3), 71.1 (C-4), 66.8 (C-5)).

2.4. ORAC assays

The ORAC values of tested compounds were obtained from two ORAC assays, including ORAC-fluorescein ($ORAC_{FL}$) and ORAC-pyrogallol red ($ORAC_{PGR}$) as previously described by López-Alarcón and Lissi [9], with some modifications. In $ORAC_{FL}$ assay, fluorescein sodium salt was used as fluorescent probe with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Stock solution of the analytes were prepared in DMSO immediately before use. Then, a reaction mixture containing fluorescein sodium salt (80 nM), and ABAP (10 mM) with or without tested compounds in 75 mM phosphate buffer (pH 7.4) was incubated at 37 °C in a multi-mode microplate reader (Filter Max F5, Molecular Devices, USA). The fluorescence intensity was measured every 2.5 min for 85 min in a microplate reader (SpectraMax 190, Molecular Devices, USA). Phosphate buffer (75 mM, pH 7.4) was used as blank. Quercetin was used as positive control, and various concentrations of Trolox (0.78–6.25 μM) were used as standards. A regression equation between Trolox concentration and the net area under curve was drawed. The $ORAC_{FL}$ values of analytes were calculated by using this regression equation, and were expressed as μmol Trolox equivalents per μmol of analyte (μmol TE/μmol).

In $ORAC_{PGR}$ assay, PGR was used as a probe with maximum absorbance at 540 nm. The reaction mixture of each well contains PGR (20 μM), and ABAP (20 mM) with or without tested compounds in phosphate buffer (75 mM, pH 7.4) [10]. The absorbance (A) was recorded immediately every 1 min for 120 min at 37 °C using a microplate reader (SpectraMax 190, Molecular Devices, USA). Phosphate buffer (75 mM, pH 7.4) was used as blank. Quercetin was used as positive control, and various concentrations of Trolox (5–100 μM) were used as standards. Values of the relative absorbance (A/A_0) were plotted as a function of time. Integration of the area under the curve was performed up to a time as the A/A_0 reached to 0.2. The net area under the curve was used for the $ORAC_{PGR}$ value calculation, and the results were expressed as μmol Trolox equivalents per μmol of analyte (μmol TE/μmol).

2.5. DPPH radical scavenging activity assay

The scavenging activities of tested compounds on 2,2-diphenyl-1-picrylhydrazyl (DPPH)-induced radicals, were measured according to the method described previously [11], with some modifications. Briefly, an alcoholic solution (80 μL) containing tested compounds was mixed with an alcoholic solution of DPPH (0.2 mM, 160 μL). The mixture was kept in dark at room temperature for 30 min, and the absorbance read at 517 nm by using a microplate reader (SpectraMax 190, Molecular Devices, USA).

2.6. Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activities of tested compounds were measured by Fe^{2+}–1,10-phenanthroline–EDTA–H_2O_2 system (Fenton reaction) according to the method of Su et al. [12], with some modifications. Briefly, 30 μL phenanthroline ethanol solution (5 mM) were added to each well containing 20 μL phosphate buffer (0.2 mM), 40 μL ferrisulphas (5 mM), 30 μL EDTA (15 mM), 40 μL H_2O_2 solution (0.1%), and 50 μL ethanol. Reaction was carried out for 60 min at 37 °C after mixing for 30 s. Then the absorbance of reaction mixture was measured at 536 nm (AP) using a microplate reader (SpectraMax 190, Molecular Devices, USA). The reaction without H_2O_2 was used as the blank (AB). When the ethanol was replaced by different concentrations of tested compounds, the absorbance of reaction mixture was recorded (AS). The radical scavenging activity was calculated according to the following equation:

$$\text{Radical scavenging activity (\%)} = (AS - AP)/(AB - AP) \times 100\%.$$