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Isolation and identification of phase I metabolites of phillyrin in rats

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ABSTRACT

Phillyrin was one of the main chemical constituents of the fruit of *Forsythia suspensa* (Thunb.) Vahl. It showed various bioactivities including antioxidant and anti-inflammatory activities. However, the metabolism of phillyrin remained unknown. This report described the isolation and identification of phase I metabolites of phillyrin in rats. Nine metabolites including six new ones were isolated by various column chromatographies and high-performance liquid chromatography. Their structures were elucidated by extensive spectroscopic analysis. The antiviral activities of phillyrin and the metabolites were evaluated against influenza A (H3N2) virus. Among them, one metabolite **M8** showed moderate activity with the IC_{50} value of 26.39 μM, and three metabolites (**M2**, **M3**, **M9**) showed weak antiviral activities at the concentration of 100 μM. Based on the structures of the metabolites, possible metabolic pathways of phillyrin in rats were also proposed.

1. Introduction

Forsythia suspensa is a well-known traditional Chinese medicine used for the treatment of pyrexia and infections [1–3]. Phillyrin, as one of the representative ingredients of *F. suspensa*, possessed various bioactivities, including anti-inflammatory [4], anti-oxidant [5,6], and antiviral activities [7]. It was also reported that phillyrin exerted anti-obesity effects in nutritive obesity mice [8]. However, pharmacokinetic studies indicated that phillyrin possessed poor absorption and low bioavailability [9,10]. Up to now, there were a limited number of studies focused on the metabolism of phillyrin in rats. In order to search for the antiviral metabolic products of phillyrin *in vivo*, we investigated the urinary metabolites of rats after oral administration of phillyrin. This paper mainly describes the isolation and identification of the phase I metabolites of phillyrin in rats, as well as the antiviral activities against influenza A (H3N2) virus.

2. Experimental

2.1. Materials

Optical rotations were measured on JASCO P-1020 spectrometer. CD spectra were recorded on JASCO J-810 spectrometer. 1D and 2D NMR spectra were measured on a Bruker AV-300/400 spectrometer. HRESIMS data were determined by a Waters Synapt G2 mass spectrometer. HP-20 macroporous resin (Diaion, Japan), ODS (50 μm, YMC, Japan), HW-40 (Tosoh, Japan) were used for open column chromatography (CC). Thin-layer chromatography (TLC) was performed using precoated silica gel plates (silica gel GF_{254},

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1 mm, Yantai). Phillyrin was supplied by Pi & pi Technology Inc. (Guangzhou, China).

2.2. Animals

Male Sprague–Dawley rats (200–250 g) were obtained from Medical Experimental Animal Center of Guangdong Province (Guangzhou, China). They were group-housed and maintained under conventional conditions at 25 °C in an alternating 12-h light/dark cycle, fed a standard laboratory chow, and allowed water *ad libitum*. Animals were fasted for 24 h prior to dosage. Phillyrin (Fig. 1) was given to rats (30 animals) by gavage at the dose of 100 mg/kg. Rats were then kept in individual metabolic cages, and urinary sample was collected after oral administration for 48 h. These urinary samples were stored at −20 °C.

2.3. Extraction and isolation

A total of 800 ml urine was collected, after condensed in vacuum, the urinary sample was chromatographed over a HP-20 macroporous resin column (5.0 × 70 cm) eluted with water, 30% EtOH–H_2O, 60% EtOH–H_2O and 95% EtOH–H_2O, successively. The 95% EtOH–H_2O eluate (D, 2.4 g) was then subjected to a ODS column (3.0 × 37 cm), eluted with MeOH–H_2O (40:60 to 80:20, v/v) in gradient to give eleven fractions. Fraction D6 (199.1 mg, 60% MeOH–H_2O eluate) was purified by preparative HPLC with 45% MeOH–H_2O as the mobile phase to yield **M1** (12.4 mg, t_R = 90.04 min), **M2** (11.0 mg, t_R = 120.71 min) and **M3** (19.4 mg, t_R = 103.27 min). Fraction D3 (651.3 mg, 50% MeOH–H_2O eluate) was subjected to a HW-40 column (3.0 × 35.0 cm) eluted with MeOH–H_2O (20:80, 40:60, 60:40, v/v), then subfractions D3B (49.9 mg, 40% MeOH–H_2O eluate), and D3F (74.1 mg, 60% MeOH–H_2O eluate) were further purified by preparative HPLC with 40% MeOH–H_2O to yield **M7** (15.0 mg, t_R = 68.80 min) and **M9** (13.0 mg, t_R = 74.97 min), and D3E (218.0 mg, 60% MeOH–H_2O eluate) was purified by preparative HPLC with 30% CH_3CN–H_2O to give **M8** (5.5 mg, t_R = 63.11 min). The 60% EtOH–H_2O eluate (C, 7.1 g) was also subjected to ODS column chromatography (3.0 × 37 cm), eluted with MeOH–H_2O (30:70–80:20, v/v) in gradient to give ten fractions, respectively. Fraction C7 (835.7 mg, 50% MeOH–H_2O eluate) was further separated by HW-40 (3.0 × 35.0 cm) eluted with MeOH–H_2O (20:80, 40:60, 60:40, v/v) and preparative HPLC (40% MeOH–H_2O) to afford **M4** (2.5 mg, t_R = 77.20 min), **M5** (11.7 mg, t_R = 76.58 min) and **M6** (1.7 mg, t_R = 82.13 min).

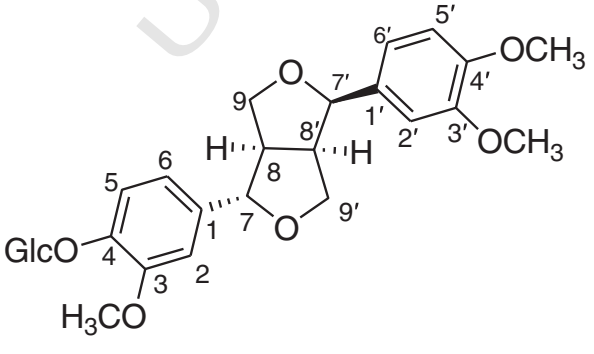

Fig. 1. The chemical structure of phillyrin.

M2 (**2**) white amorphous powder; $[\alpha]_D^{20}$ +28.0 (*c* 0.25 MeOH); UV (MeOH) λ_{max} (log ε): 205 (4.32), 230 (3.85), 280 (3.60) nm; CD (MeOH, c 0.4): 238 nm ($\Delta\varepsilon$ 0.39), 285 nm ($\Delta\varepsilon$ −0.31); IR (KBr) ν_{max}: 3434, 2962, 2861, 2834, 1592 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS m/z 395.1460 $[M + Na]^+$ (calcd for $C_{21}H_{24}O_6Na$, 395.1471).

M3 (**3**) white amorphous powder; $[\alpha]_D^{20}$ +17.6 (c 0.25, MeOH); UV (MeOH) λ_{max} (log ε): 206 (4.84), 232 (4.23), 278 (3.55) nm; CD (MeOH, c 0.2): 236 nm ($\Delta\varepsilon$ 0.33), 297 nm ($\Delta\varepsilon$ −0.18); IR (KBr) ν_{max}: 3417, 2963, 2922, 2847, 1707 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS m/z 425.1566 $[M + Na]^+$ (calcd for $C_{22}H_{26}O_7Na$, 425.1576).

M4 (**4**): white amorphous powder; $[\alpha]_D^{20}$ +49.3 (c 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 206 (4.51), 230 (3.86), 280 (3.12) nm; CD (MeOH, c 0.3): 226 nm ($\Delta\varepsilon$ 0.72), 291 nm ($\Delta\varepsilon$ −0.20); IR (KBr) ν_{max}: 3412, 2965, 2917, 2856, 1702 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS m/z 411.1417 $[M + Na]^+$ (calcd for $C_{21}H_{24}O_7Na$, 411.1420).

M5 (**5**): white amorphous powder; $[\alpha]_D^{20}$ +43.3 (c 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 206 (4.18), 230 (3.61), 280 (3.06) nm; CD (MeOH, c 0.3): 231 nm ($\Delta\varepsilon$ 1.70), 280 nm ($\Delta\varepsilon$ −0.16); IR (KBr) ν_{max}: 3408, 2962, 2922, 2851, 1602 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS m/z 397.1625 $[M + Na]^+$ (calcd for $C_{21}H_{26}O_6Na$, 397.1627).

M6 (**6**): white amorphous powder; $[\alpha]_D^{20}$ +18.0 (c 0.3, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.33), 226 (3.97), 279 (3.70) nm; CD (MeOH, c 0.5): 230 nm ($\Delta\varepsilon$ 1.89), 272 nm ($\Delta\varepsilon$ −0.05); IR (KBr) ν_{max}: 3450, 2928, 2859, 2358,1642 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS m/z 397.1638 $[M + Na]^+$ (calcd for $C_{21}H_{26}O_6Na$, 397.1627).

M7 (**7**): white amorphous powder; $[\alpha]_D^{20}$ +66.4 (c 0.25, MeOH); UV (MeOH) λ_{max} (log ε): 206 (4.45), 230 (3.83), 278 (2.83) nm; CD (MeOH, c 0.4): 230 nm ($\Delta\varepsilon$ 0.93), 290 nm ($\Delta\varepsilon$ −0.17); IR (KBr) ν_{max}: 3425, 2962, 2922, 2847, 1602 cm^{-1}; ^1H and ^{13}C NMR data (see Tables 1 and 2); HRESIMS m/z 427.1728 $[M + Na]^+$ (calcd for $C_{22}H_{28}O_7Na$, 427.1733).

2.4. Antiviral activity assay

The antiviral activities of phillyrin, **M1**–**M3**, **M5**, **M7**–**M9** and favipiravir (T-705) against influenza A (H3N2) virus were evaluated *in vitro*. Antiviral activity was determined by the inhibition of virus-induced cytopathic effect (CPE) assay [11]. The concentration reducing CPE by 50% compared with virus control was defined as IC_{50}. The CPE and cytotoxicity were quantified by CellTiter-Glo® luminescent cell viability assay, and the IC_{50} and TC_{50} value was calculated by Origin 8.0. According to the results, **M8** showed moderate antiviral activity against influenza A (H3N2) virus with an IC_{50} value of 26.39 μM, and selective index (SI = TC_{50}/IC_{50}) value of 7.93. In addition, **M2**, **M3** and **M9** exhibited inhibitory activity against viral-induced CPE at the concentration of 100 μM.

3. Result and discussion

Nine metabolites were isolated from rat's urine after oral administration of phillyrin using various chromatographic methods. Among them, **M2** to **M7** were new metabolites. All

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