METHOXYFLAVONES FROM ORTHOSIPHON STAMINEUS AND THEIR PTP1B INHIBITORY ACTIVITIES

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ABSTRACT

Phytochemical analysis of the methanol extract of the aerial parts of Orthosiphon stamineus Benth. led to the isolation of four flavone compounds including 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (1), 3,5,7,3′,4′-pentamethoxyflavone (2), 3,3′-dihydroxy-5,7,4′-trimethoxyflavone (3), and 3,5,3′-trihydroxy-7,4′-dimethoxyflavone (4). Their chemical structures were determined from the spectroscopic evidences, including 1D-NMR and MS, respectively. The inhibitory effects of the isolates (1-4) against protein tyrosine phosphatase 1B (PTP1B) were investigated in vitro using ursolic acid as positive control. Among the isolates, compound 4 exhibited potential activity with IC₅₀ value of 10.12 ± 0.19 μM, the others showed weak activity. In this assay, ursolic acid displayed an IC₅₀ value of 3.42 ± 0.25 μM. This is indicated that 3,3′-dihydroxy-5,7,4′-trimethoxyflavone (4) may be useful for discovery of PTP1B inhibitors as antidiabetic agent.

Keywords: Orthosiphon stamineus Benth., flavone, ursolic acid, PTP1B inhibitor, type 2 diabetes.

1. INTRODUCTION

Nowadays diabetes is a huge and growing problem. The most recent estimate in 2017 shows that 425 million people are living with diabetes and this number is set to rise beyond 625 million in less than 25 years [1]. Type 2 diabetes (T2D), or noninsulin-dependent diabetes mellitus, is the most common type accounting for approximately 90% of the total cases among the three types of diabetes [2]. This type is characterized by a resistance to insulin, a peptide hormone produced by β-cells in the pancreas, which is responsible for glucose homeostasis [3, 4]. The insulin signaling pathway is negatively regulated by protein tyrosine phosphatases, most notably, protein tyrosine phosphatase 1B (PTP1B) [4]. The overexpression of PTP1B has been shown to inhibit the increased expression of insulin in insulin-resistant states [5]. Furthermore, recent genetic evidence has shown that PTP1B gene variants are associated with changes in
insulin sensitivity [6]. At the genetic, molecular, biochemical, and physiological levels, PTP1B seems to be a promising drug target for the treatment of T2D and at-risk obese patients [7]. Natural products are rich sources of novel active agents for clinical uses [8]. Previous reports indicate that there are more than 1000 plant species being used to treat T2D all over the world [3] and various natural compounds display PTP1B inhibitory activity [9].

Orthosiphon stamineus Benth., belonging to Lamiaceae family, has common name as Cat’s Whiskers, Java Tea in America, Kumis Kuching in Indonesia, Misai Kuching in Malaysia, and “Râu Mèo” in Viet Nam. This is a fast-growing herbaceous shrub that can reach 1-2 meters tall and spread to a meter wide. The plant produces racemes that are 10-20 cm long with pretty tubular flowers that are uniquely shaped and bear 5-6 cm long stamens that look like cat’s whiskers, hence the common name. The plant is grown throughout Southeast Asia, Australia, and also Africa [10]. Traditional uses have trusted for many centuries for treating ailments of the kidney, bladder stone, urinary tract infection, liver and bladder problems, rheumatism, diabetes, and gout. In Viet Nam, it has been used for many decades in the treatment of renal inflammation, kidney stones and dysuria. The aerial parts are used as tea to reduce cholesterol and blood pressure. However, to the best of our knowledge, the chemical constituents of this plant have not been reported in detail. Therefore, in the interest of promoting drug discovery from natural sources, this research was conducted to identify bioactive compounds from the aerial parts of O. stamineus, focusing on PTP1B inhibitory activity.

2. MATERIALS AND METHODS

2.1. General experimental procedures

The ¹H-NMR (500 MHz) and ¹³C-NMR (125 MHz) spectra were recorded on a Bruker AM500 FT-NMR spectrometer, TMS was used as an internal standard. The electrospray ionization mass spectra (ESI-MS) were obtained on an Agilent 1260 series single quadrupole LC/MS system. Column chromatography (CC) was performed on silica gel (Kieselgel 60, 70-230 mesh and 230-400 mesh, Merck) and YMC RP-18 resins (30-50 μm, Fuji Silysia Chemical Ltd.). Thin layer chromatography (TLC) used pre-coated silica gel 60 F254 (1.05554.0001, Merck) and RP-18 F254S plates (1.15685.0001, Merck). Compounds were visualized by spraying with aqueous 10 % H₂SO₄ and heating for 3–5 minutes.

2.2. Plant materials

The aerial parts of Orthosiphon stamineus Benth. were collected in January, 2017 at Ngu Hiep, Thanh Tri, Ha Noi. The sample was identified by Dr. Nguyen Quoc Binh (Viet Nam National Museum of Nature, VAST). A voucher specimen (SH-164) was deposited at the Institute of Natural Products Chemistry (INPC), VAST.

2.3. Extraction and isolation

The dried aerial parts of O. stamineus (2.1 kg) were cut into small pieces (1 to 2 cm long) before extracted with MeOH under sonication for 10 h, at 45 °C, each 5 L for 4 times. The MeOH-soluble extract was dried under reduced pressure to give a crude MeOH-extract (196.4 g). This crude extract was excessively fractionated with hexane and EtOAc to give the hexane (26 g) and EtOAc (11 g) fractions after vacuum evaporating under reduced pressure. The EtOAc fraction was further subjected on a silica gel column chromatography (10 × 60 cm I.D; 63–200
μm particle size), using a gradient solvent system of hexane:acetone (15:1 → 0:1, v/v), to yield ten combined fractions (OS.EA1 to OS.EA10) according to their TLC profiles. Fraction OS.EA2 was further chromatographed on a silica gel column (3.5 × 60 cm), eluting with hexane:EtOAc (10:1 to 5:1, v/v) to give five subfractions (OS.EA2.1 to OS.EA2.5). Compounds 1 (50 mg) and 2 (13.2 mg) were purified from subfraction OS.EA2.3 by a C18 reversed-phase (RP-18) chromatography column (2.0 × 60 cm; 40–63 μm particle size) and eluted with a gradient solvent system of MeOH–H2O (from 6:4 to 8:2, v/v). Fraction OS.EA4 was also rechromatographed on a silica gel column (3.5 × 60 cm), eluting with hexane:EtOAc (6:1 to 1:1, v/v) to give ten subfractions (OS.EA4.1 to OS.EA4.10). Subfraction OS.EA4.7 was further chromatographed by a reversed-phase column (2.0 × 80 cm) using reversed-phase (RP-C18) silica gel and eluting with MeOH–H2O gradient mixture (from 1:1.5 to 3:1, v/v), afforded compounds 3 and 4, respectively.

5-hydroxy-3,7,3′,4′-tetramethoxyflavone (1): Yellow amorphous powder; 1H-NMR (500 MHz, CDCl3) δH (ppm) 6.87 (1H, br s, H-6), 6.78 (1H, br s, H-8), 7.63 (1H, d, J = 2.0 Hz, H-2′), 7.15 (1H, d, J = 8.4 Hz, H-5′), 7.71 (1H, dd, J = 2.0, 8.4 Hz, H-6′), 3.80 (3H, s, 3-OCH3), 3.98 (3H, s, 7-OCH3), 3.93 (3H, s, 3′-OCH3), 3.96 (3H, s, 4′-OCH3), 12.95 (1H, s, 5-OH); 13C-NMR (125 MHz, CDCl3) δC (ppm) 152.3 (C-2), 132.8 (C-3), 182.8 (C-4), 158.9 (C-5), 90.8 (C-6), 164.8 (C-7), 90.8 (C-8), 149.5 (C-9), 106.3 (C-10), 123.9 (C-1′), 111.3 (C-2′), 153.3 (C-3′), 153.4 (C-4′), 108.9 (C-5′), 120.3 (C-6′), 61.1 (3-OCH3) 56.5 (7-OCH3), 56.3 (3′-OCH3), 56.3 (4′-OCH3).

3,5,7,3′,4′-pentamethoxyflavone (2): Yellow amorphous powder; 1H-NMR (500 MHz, acetone-d6) δH (ppm) 7.11 (1H, br s, H-6), 6.58 (1H, br s, H-8), 7.57 (1H, d, J = 2.0 Hz, H-2′), 7.13 (1H, d, J = 8.4 Hz, H-5′), 7.64 (1H, dd, J = 2.0, 8.4 Hz, H-6′), 3.82 (3H, s, 3-OCH3), 3.88 (3H, s, 5-OCH3), 4.00 (3H, s, 7-OCH3), 3.91 (3H, s, 3′-OCH3), 3.95 (3H, s, 4′-OCH3); 13C-NMR (125 MHz, CDCl3) δC (ppm) 153.8 (C-2), 134.8 (C-3), 181.2 (C-4), 159.6 (C-5), 95.8 (C-6), 164.1 (C-7), 92.5 (C-8), 158.0 (C-9), 107.3 (C-10), 123.4 (C-1′), 111.3 (C-2′), 153.4 (C-3′), 153.1 (C-4′), 110.9 (C-5′), 121.1 (C-6′), 61.1 (3-OCH3), 56.1 (5-OCH3), 56.3 (7-OCH3), 56.5 (3′-OCH3), 56.7 (4′-OCH3).

3,5,3′-trihydroxy-7,4′-dimethoxyflavone (3): Yellow amorphous powder; 1H-NMR (500 MHz, acetone-d6) δH (ppm) 6.90 (1H, br s, H-6), 6.66 (1H, br s, H-8), 7.52 (1H, d, J = 2.0 Hz, H-2′), 7.14 (1H, d, J = 8.5 Hz, H-5′), 7.58 (1H, dd, J = 2.0, 8.5 Hz, H-6′), 8.09 (1H, s, 3-OH), 12.67 (1H, s, 5-OH), 4.00 (3H, s, 7-OCH3), 3.94 (3H, s, 4′-OCH3); 13C-NMR (125 MHz, acetone-d6) δC (ppm) 164.9 (C-2), 131.1 (C-3), 183.5 (C-4), 165.0 (C-5), 91.7 (C-6), 154.9 (C-7), 104.4 (C-8), 157.2 (C-9), 106.5 (C-10), 125.1 (C-1′), 113.6 (C-2′), 147.9 (C-3′), 151.7 (C-4′), 112.5 (C-5′), 119.7 (C-6′), 56.9 (7-OCH3), 56.5 (4′-OCH3).

3,3′-dihydroxy-5,7,4′-trimethoxyflavone (4): Yellow amorphous powder; 1H-NMR (500 MHz, CDCl3) δH (ppm) 7.12 (1H, br s, H-6), 6.51 (1H, br s, H-8), 7.48 (1H, d, J = 2.0 Hz, H-2′), 7.13 (1H, d, J = 8.4 Hz, H-5′), 7.52 (1H, dd, J = 2.0, 8.4 Hz, H-6′), 3.87 (3H, s, 5-OCH3), 3.94 (3H, s, 7-OCH3), 4.01 (3H, s, 4′-OCH3), 12.95 (1H, s, 5-OH); 13C-NMR (125 MHz, CDCl3) δC (ppm) 152.3 (C-2), 132.8 (C-3), 182.8 (C-4), 158.9 (C-5), 90.8 (C-6), 164.8 (C-7), 90.8 (C-8), 149.5 (C-9), 106.3 (C-10), 123.9 (C-1′), 111.3 (C-2′), 153.3 (C-3′), 153.4 (C-4′), 108.9 (C-5′), 120.3 (C-6′), 61.1 (3-OCH3) 56.5 (7-OCH3), 56.3 (3′-OCH3), 56.3 (4′-OCH3).

2.4. Protein tyrosine phosphatase IB (PTP1B) inhibitory assay

Protein tyrosine phosphatase IB (human recombinant) was purchased from Biomol International LP, Plymouth Meeting, PA, USA, and the inhibitory activities of the tested samples were evaluated using the method described in the reported paper [11].
3. RESULTS AND DISCUSSION

3.1. Isolation and structural elucidation of isolated compounds

The methanol extract of the aerial parts of Cat’s whiskers were partitioned with hexane and ethyl acetate. Phytochemical research of the ethyl acetate fraction led to the isolation of four natural products (1-4) (Fig. 1).

![Chemical structure of compounds 1-4 isolated from O. stamineus Benth.](image)

**Figure 1.** Chemical structure of compounds 1-4 isolated from O. stamineus Benth.

Compound 1 was obtained as yellow powder, the ESI mass spectrum of 1 exhibited an ion peak at m/z 359 [M+H]+, suggesting a molecular formula of C_{19}H_{20}O_{7} (M = 358). Its UV spectrum showed absorption bands of a typical flavone. The difference between (1H, δ = 3.88 (1H, br s, H-6)), 3.93 (3H, s, 7-OCH_{3}), and 3.96 (3H, s, 4′-OCH_{3}), with corresponding carbon signals at δ_c 61.1 (3-OCH_{3}), 56.5 (7-OCH_{3}), 56.3 (3′-OCH_{3}), and 56.3 (4′-OCH_{3}), all of these were found to be attached to C-3, C-7, C-3′, and C-4′ due to an conjugated hydroxyl group (δ_H 12.95, 1H, s) attached at C-5 in the ¹H NMR spectrum [13]. A detailed comparison between the ¹H and ¹³C-NMR data of 1 with published values led to the structurally identification of 1 as 5-hydroxy-3,7,3′,4′-tetramethoxyflavone [14].

Compound 2 was also obtained as yellow powder. A molecular ion peak at m/z 373.12 [M+H]+ obtained in the ESI-MS revealing a molecular formula of C_{20}H_{20}O_{7} for 2. The ¹H- and ¹³C-NMR spectra of compound 2 were quite similar to compound 1 with four methoxy groups at δ_H 3.82 (3-OCH_{3}), 4.00 (7-OCH_{3}), 3.91 (3′-OCH_{3}), and 3.95 (4′-OCH_{3}), two singlet proton peaks at δ_H 7.11 (H-6) and 6.58 (H-8) of ring A, and an ABX-aromatic spin system of ring B at δ_H 7.57 (1H, d, J = 2.0 Hz, H-2′). The difference between 1 and 2 was only the replacement of the 5-OH group in 1 by 5-OCH_{3} group in 2 (δ_H 3.88 and δ_c 56.1). Thus, chemical structure of compound 2 was determined as 3,5,7,3′,4′-pentamethoxyflavone [15].
Compound 3 was obtained as yellow amorphous powder. The molecule formula of 3 was revealed as C_{13}H_{15}O_{7} based on a molecular ion peak at m/z 331.07 [M+H]^+ obtained from its ESI-MS. The $^1$H-NMR spectrum of 3 also showed an aromatic ABX-spin system at $\delta_H$ 7.58 (1H, dd, $J = 2.0$, 8.5 Hz, H-6'), 7.14 (1H, d, $J = 8.5$ Hz, H-5'), and 7.52 (1H, d, $J = 2.0$ Hz, H-2') assigning for the B ring, two broad singlet proton peaks at $\delta_H$ 6.90 (1H, br s, H-6) and 6.66 (1H, br s, H-8) of the A ring, and two singlet proton resonated at $\delta_H$ 12.94 (1H, s), which was assignable to 5-OH, and $\delta_H$ 8.09 (1H, br s) assignable to 3-OH [13]. In addition, two methoxy protons at $\delta_H$ 4.00 and 3.94 (each 3H, s) with corresponding carbons at $\delta_C$ 56.9 and 56.5 were displayed in the $^1$H- and $^{13}$C-NMR spectra of 3. The chemical shifts of C-3' ($\delta_C$ 147.9) and C-4' ($\delta_C$ 151.7) in the $^{13}$C NMR spectrum revealed oxygenation at these carbons. In addition, the chemical shifts of C-3 appeared at $\delta_C$ 131.1 in the $^{13}$C-NMR, revealing a hydroxy group attached at C-3 position. Analysis of the HMBC data of 3 allowed us to assign the attachment of two methoxy group at C-7 and C-4', respectively (Figure 2). Thus, the structure of compound 3 was established as 3,5,3'-trimethoxy-7,4'-dimethoxyflavone [16].

Compound 4 was also obtained as yellow powder. A molecular ion peak at m/z 345.09 [M+H]^+ was observed in the ESI-MS suggesting its molecular formula of C_{14}H_{17}O_{7}. The $^1$H- and $^{13}$C-NMR spectra of compound 4 were quite similar to that of compound 3 except only for an additional methoxy signals at $\delta_H$ 3.87 and $\delta_C$ 56.3 in 4. In addition, the conjugated hydroxyl peak in the $^1$H NMR spectrum of 3 was disappeared in 4. Two methoxy groups at $\delta_H$ 4.01 (7-OCH$_3$) and 3.94 (4'-OCH$_3$), two singlet proton peaks at $\delta_H$ 7.12 (H-6) and 6.51 (H-8) of ring A, an ABX-aromatic spin system of ring B at $\delta_H$ 7.48 (1H, d, $J = 2.0$ Hz, H-2'), 7.12 (1H, d, $J = 8.5$ Hz, H-5'), and 7.52 (1H, dd, $J = 2.0$, 8.5 Hz, H-6') were also presented. Thus, chemical structure of compound 4 was determined as 3,3'-dihydroxy-7,4'-dimethoxyflavone [17].

![Figure 2](image-url)  
Figure 2. $^1$H-$^{13}$C (→) key HMBC correlations of compounds 3 and 4.

### 3.2. PTP1B inhibitory activity of isolated compounds

The inhibitory effects of isolated compounds (1-4) on PTP1B enzyme activity were measured using ursolic acid as positive control (Table 1) [11]. All of the isolates (1-4) exhibited dose-dependent inhibition, among the isolates, 3,3'-dihydroxy-5,7,4'-trimethoxyflavone (4) possessed potential inhibitory activity with an IC$_{50}$ value of 10.12 ± 0.19 µM. Compound 3 displayed weak activity with IC$_{50}$ value of 52.64 ± 4.12 µM while compounds 1 and 2 showed no effect. The positive control, ursolic acid, showed an IC$_{50}$ value of 3.42 ± 0.07 µM in this enzyme assay. Among these isolates, compound 1 with four methoxy groups at C-3, C-7, C-3', and C-4', and a hydroxy group at C-5 showed no activity (IC$_{50}$ > 100 µM), compound 2 with five methoxy group exhibited the same manner. In contrast, compound 3 with three hydroxyl groups at C-3, C-5, and C-3', compound 4 with two hydroxyl groups at C-3 and C-3' displayed stronger activity (IC$_{50}$ value of 52.64 ± 4.12 and 10.12 ± 0.19 µM). This observation may suggest that the number of methoxy group and/or the position of the substitution of methoxy by hydroxy group in these flavonol-type compounds may be responsible to the diminishment of
inhibitory activity of these compounds on PTP1B. In our knowledge, compounds 1-4 were first time isolated from O. stamineus, and that the PTP1B inhibitory activities of these compounds have also been investigated for the first time.

Table 1. PTP1B inhibitory activity of isolated compounds (1-4) and ursolic acid.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Inhibitory activity (IC$_{50}$, µM)$^a$</th>
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<tbody>
<tr>
<td>5-hydroxy-3,7,3',4'-tetramethoxyflavone (1)</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>3,5,7,3',4'-pentamethoxyflavone (2)</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>3,5,3'-trihydroxy-7,4'-dimethoxyflavone (3)</td>
<td>10.12 ± 0.19</td>
</tr>
<tr>
<td>3,3'-dihydroxy-5,7,4'-trimethoxyflavone (4)</td>
<td>52.64 ± 4.12</td>
</tr>
<tr>
<td>Ursolic acid $^b$</td>
<td>3.42 ± 0.07</td>
</tr>
</tbody>
</table>

$^a$ Results are expressed as IC$_{50}$ values (µM), determined by regression analysis and expressed as the means ± SD of three replicates.

$^b$ Positive control.

4. CONCLUSIONS

Using combined chromatographic and spectroscopic methods, four flavones including 5-hydroxy-3,7,3',4'-tetramethoxyflavone (1), 3,5,7,3',4'-pentamethoxyflavone (2), 3,5,3'-trihydroxy-7,4'-dimethoxyflavone (3), and 3,3'-dihydroxy-5,7,4'-trimethoxyflavone (4) were isolated and structurally identified from the methanol extract of the aerial parts of Orthosiphon stamineus Benth. All of the isolates (1-4) were investigated for their inhibitory effects on PTP1B enzyme activity using an in vitro assay, among them, 3,3'-dihydroxy-5,7,4'-trimethoxyflavone (4) possessed potential activity with an IC$_{50}$ value of 10.12 ± 0.19 µM. Compound 3 displayed weak activity with IC$_{50}$ value of 52.64 ± 4.12 µM while compounds 1 and 2 showed no effect. Ursolic acid as positive control showed an IC$_{50}$ value of 3.42 ± 0.07 µM in this enzyme assay.

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