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PTP1B inhibitory flavonols from *Orthosiphon stamineus* Benth.

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Abstract

From the alcoholic extract of the aerial parts of *Orthosiphon stamineus* Benth., three flavonols have been isolated. Their chemical structures were elucidated to be 5,7,3',4'-tetramethylquercetin (**1**), 3'-hydroxy-3,5,7,4'-tetramethoxyflavone (**2**), and 3,5-dihydroxy-7,3',4'-trimethoxyflavone (**3**), by combining spectroscopic data and physicochemical data analyses (including IR, UV, NMR and MS). Compounds **1**-**3** exhibited potential PTP1B inhibitory activities with IC_{50} values of 8.92±0.72, 22.25±1.70, and 52.64±4.12 μM, respectively. The positive control, ursolic acid, showed an IC_{50} value of 3.42±0.97 μM in this assay.

Keywords. *Orthosiphon stamineus* Benth., PTP1B inhibitor, flavonol, Cat's whiskers, flavonoid.

1. INTRODUCTION

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 [1]. 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 [2, 3]. The insulin signaling pathway is negatively regulated by protein tyrosine phosphatases, most notably, protein tyrosine phosphatase 1B (PTP1B) [3]. The PTP1B over-expression inhibited the increased expression of insulin in insulin-resistant states [4], while PTP1B knockout mice display increased insulin sensitivity and show lower weight gain when consuming normal and high-fat diets [5]. PTP1B-deficient mice also show decreased leptin levels and hypersensitivity to leptin compared with wild-type littermates on low- and high-fat diets [6]. Thus, PTP1B inhibitors could be useful in treating type 2 diabetes as well as obesity [7, 8].

Orthosiphon stamineus Benth., belonging to Lamiaceae family, has a common name as Cat's Whiskers, Java Tea in America, Kumis Kuching in Indonesia, and Misai Kuching in Malaysia. The plant is grown throughout Southeast Asia, Australia, and also Africa [9]. 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 Vietnam, it has been used for many decades in the treatment of renal inflammation, kidney stones and dysuria. The aerial parts are used as a tea to reduce cholesterol and blood pressure. In this paper, the isolation, structural elucidation, and the PTP1B inhibitory effects of the compounds isolated from *O. stamineus* are reported.

2. EXPERIMENTAL

2.1. General experimental procedures

The ^{1}H-NMR (500 MHz) and ^{13}C-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 F_{254} (1.05554.0001, Merck) and RP-18 F_{254S} plates (1.15685.0001, Merck). Compounds were visualized by spraying with aqueous 10% H_2SO_4 and heating for 3-5 minutes.

2.2. Plant material

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

2.3. Extraction and isolation

The dried aerial parts of *O. stamineus* (2.1 kg) were cut into small pieces before extraction 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 *n*-hexane and EtOAc to give the *n*-hexane (26 g) and EtOAc (11 g) fractions after solvent evaporation under reduced pressure. The EtOAc fraction was further subjected to a silica gel column chromatography (10 × 80 cm I.D; 63–200 µm particle size), using a gradient solvent system of *n*-hexane:acetone (10:1 → 0:1, v/v), to yield ten combined fractions (OS.E1 to OS.E10) according to their TLC profiles. Fraction OS.E3 was chromatographed on a silica gel column (3.5 x 60 cm), eluting with *n*-hexane:EtOAc (8:1 to 2:1, v/v) to give five subfractions (OS.E3.1 to OS.E3.5). Compound **1** was purified from subfraction OS.E3.3 by a C_{18} reversed-phase (RP-18) chromatography column (2.0 × 60 cm; 40–63 µm particle size) and eluted with ACN–H_2O (1.5:1, v/v). Subfraction OS.E3.2 was chromatographed on a column (2.0 × 120 cm) using Sephadex LH-20 resin and eluting with MeOH–H_2O gradient mixture (from 2:1 to 3:1, v/v) to afford compounds **2** and **3**.

2.4. Protein tyrosine phosphatase 1B (PTP1B) inhibitory assay

Protein tyrosine phosphatase 1B (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 as described [10].

3. RESULTS AND DISCUSSION

The methanol extract of the aerial parts of Cat's whiskers was partitioned with *n*-hexane and ethyl acetate. Phytochemical research of the ethyl acetate fraction led to the isolation of three natural compounds (**1-3**) (Fig. 1).

Compound **1** was isolated as a yellow powder, the EI mass spectrum of **1** exhibited an ion peak at *m/z* 359 $[M+H]^+$, corresponding to the molecular formula of $C_{19}H_{18}O_7$, M = 358. Its UV spectrum showed absorption bands of a typical flavonol at 270 and 340 nm [11]. The ^{1}H NMR spectrum of **1** showed two broad *singlet* proton peaks at δ_H 7.14 (H-6) and 6.52 (H-8) that helped define ring A. An ABX-aromatic spin system [δ_H 7.52 (1H, dd, J = 2.0, 8.5 Hz, H-6'), 7.12 (1H, d, J = 8.5 Hz, H-5'), and 7.48 (1H, d, J = 2.0 Hz, H-2')], was consistent with the substitution pattern assigned for ring B. The chemical shifts of C-3' (δ_C 147.9) and C-4' (δ_C 153.3) in the ^{13}C NMR spectrum revealed oxygenation at these carbons. In addition, the ^{1}H and ^{13}C NMR spectra of **1** gave four methoxy groups (Table 1), all of these were found to be attached to C-5, C-7, C-3', and C-4' by analyzing its HMBC data (Fig. 2). A detailed comparison between the ^{1}H and ^{13}C NMR data of **1** with published values led to the structural identification of **1** as 5,7,3',4'-tetramethylquercetin [12].

Compound **2** was also obtained as a yellow powder. Its molecular formula was deduced as $C_{19}H_{18}O_7$ from a molecular ion peak at *m/z* 359 $[M + H]^+$ in the EI-MS. The ^{1}H and ^{13}C NMR spectra of compound **2** were quite similar to compound **1** with four methoxy groups at δ_H 3.85 (s), 3.89 (s), 3.90 (s), and 3.93 (s), two *singlet* proton peaks at δ_H 6.73 (H-6) and 6.51 (H-8) of ring A, and an ABX-aromatic spin system of ring B at δ_H 7.42 (1H, dd, J = 2.0, 8.5 Hz, H-6'), 6.89 (1H, d, J = 8.5 Hz, H-5'), and 7.23 (1H, d, J = 2.0 Hz, H-2') (Table 1). Analysis of its HMBC data led to the establishment of the linkage of methoxy groups at C-3, C-5, C-7 and C-4' (Fig. 2). Thus, the chemical structure of compound **2** was determined as 3'-hydroxy-3,5,7,4'-tetramethoxyflavone [13].

Compound **3** was obtained as a yellow amorphous powder, and its UV spectrum showed absorption maxima of a typical flavonol at 272 and 338 nm [11]. The molecular formula of **3** was established as $C_{18}H_{16}O_7$ for **3** based on the molecular ion peak at *m/z* 345 $[M+H]^+$ obtained from its EI-MS. The ^{1}H NMR spectrum of **3** also showed an aromatic ABX-spin system at δ_H 7.93 (1H, br d, J = 8.5 Hz, H-6'), 6.93 (1H, d, J = 8.5 Hz, H-5'), and 7.45 (1H, br s, H-2') assigning for the B ring, two broad *singlet* proton peaks at δ_H 6.57 (H-6) and 6.53 (H-8) of the A ring, and a *singlet* proton resonated at δ_H 12.94 (1H, s), which was assignable to 5-OH [14]. In addition, three methoxy protons at δ_H 3.97, 3.95, and 3.90 (each 3H, s) with corresponding carbons at δ_C 60.6, 56.5, and 56.9 were displayed in the ^{1}H and ^{13}C NMR spectra of **3**. The chemical

shifts of C-3' (δ_C 147.9) and C-4' (δ_C 154.9) in the ^{13}C NMR spectrum revealed oxygenation at these carbons. In addition, the chemical shifts of C-3 respectively appeared at δ_C 131.1 in the ^{13}C NMR, revealing a hydroxyl group attached to C-3 position. Thus, compound **3** was identified as 3,5-dihydroxy-7,3',4'-trimethoxyflavone [15].

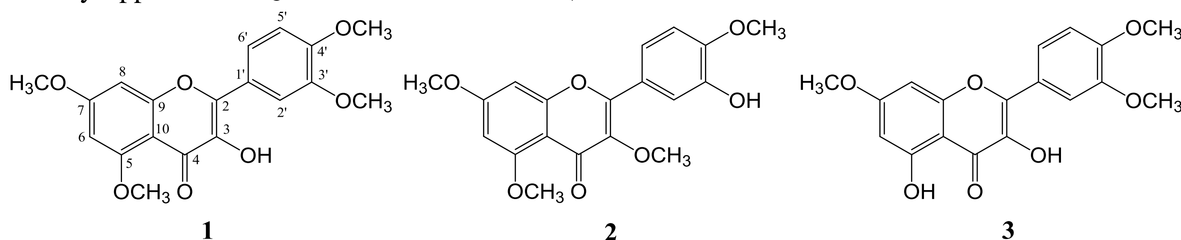

Figure 1: Chemical structure of compounds **1**-**3** isolated from *O. stamineus* Benth.

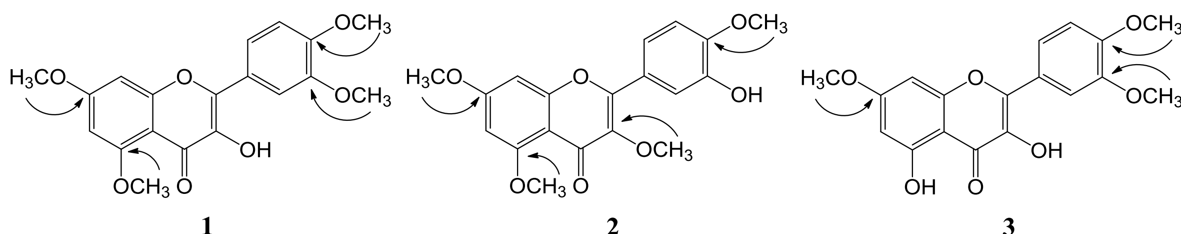

Figure 2: ^1H- ^{13}C key HMBC correlations of compounds **1**-**3**

Table 1: ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data of compounds **1**-**3**

Position	**1**[a]		**2**[b]		**3**[b]	
	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
2		151.3		152.5		151.7
3		141.4		140.3		131.1
4		176.6		177.2		179.7
5		158.9		157.7		166.1
6	7.14 (br s)	107.4	6.73 (s)	107.3	6.57 (br s)	100.0
7		161.7		161.1		161.7
8	6.52 (br s)	97.8	6.51 (s)	96.3	6.53 (br s)	94.9
9		155.4		151.8		159.2
10		113.6		112.8		105.9
1′		125.3		124.0		122.9
2′	7.48 (d, 2.0)	113.4	7.23 (d, 2.0)	111.1	7.45 (br s)	116.2
3′		147.9		154.5		147.9
4′		153.3		149.2		154.9
5′	7.12 (d, 8.5)	112.5	6.89 (d, 8.5)	108.5	6.93 (d, 8.5)	112.2
6′	7.52 (dd, 2.0, 8.5)	119.2	7.42 (dd, 2.0, 8.5)	119.6	7.93 (br d, 8.5)	118.4
3-OCH_3			3.85 (s)	56.1		
5-OCH_3	3.94 (s)	61.5	3.89 (s)	61.6		
7-OCH_3	4.01 (s)	62.4	3.93 (s)	62.2	3.97 (s)	60.6
3′-OCH_3	3.87 (s)	56.8			3.90 (s)	56.9
4′-OCH_3	3.82 (s)	56.5	3.90 (s)	56.4	3.95 (s)	56.5
3-OH	8.07 (br s)				8.09 (s)	
5-OH					12.94 (s)	

[a] Measured in acetone-d_6, [b] Measured in $CDCl_3$.

The inhibitory effects of isolated compounds **1**-**3** on PTP1B enzyme activity were measured using ursolic acid as positive control (table 2) [13]. All of the isolates (**1**-**3**) exhibited potential inhibitory activities on PTP1B enzyme with an IC_{50} value of 8.92±0.72, 22.25±1.70, and 52.64±4.12 μM. The positive control, ursolic acid, showed an IC_{50} value of 3.42±0.97 μM in this enzyme assay. Among these

isolates, compound **1** with four methoxy groups at C-5, C-7, C-3′, and C-4′ showed the strongest inhibition (IC_{50} = 8.92±0.72 μM), while compound **2** with exchanged hydroxy and methoxy group between C-3 and C-3' showed less activity (IC_{50} = 22.25±1.70 μM). Compound **3** with loss of one methoxy moiety, as compared with **1** and **2**, exhibited weaker activity with IC_{50} = 52.64±4.12 μM. This observation suggested that the number of methoxy groups 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 enzyme activity.

Table 2: PTP1B inhibitory activity of isolated compounds (**1**-**3**) and ursolic acid

Compounds	Inhibitory activity (IC_{50}, μM)[a]
5,7,3',4'-tetramethylquercetin (**1**)	8.92±0.72
3'-hydroxy-3,5,7,4′-tetramethoxyflavone (**2**)	22.25±1.70
3,5-dihydroxy-7,3',4'-trimethoxyflavone (**3**)	52.64±4.12
Ursolic acid[b]	3.42±0.97

[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. CONCLUSION

Using combined chromatographic and spectroscopic methods, three flavonols including 5,7,3',4'-tetramethylquercetin (**1**), 3'-hydroxy-3,5,7,4'-tetramethoxyflavone (**2**), and 3,5-dihydroxy-7,3',4'-trimethoxyflavone (**3**) were isolated and structurally identified from the methanol extract of the aerial parts of *O. stamineus* Benth. All of the isolates (**1**-**3**) exhibited potential inhibitory effects on PTP1B enzyme activity with IC_{50} values of 8.92±0.72, 22.25±1.70, and 52.64±4.12 μM, respectively, while ursolic acid, used as positive control, showed an IC_{50} value of 3.42±0.97 μM in this enzyme assay. To the best of our knowledge, this is the first time that these flavonols have been isolated from this species and that the PTP1B inhibitory activity of these compounds has also been reported for the first time.

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