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# Secondary Metabolites with Tyrosinase and Acetylcholinesterase Inhibitory Activities from Leonuri Fructus

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**Abstract:** Fourteen secondary metabolites, including one cinnamate derivative (**1**), three spirocyclic nortriterpenoids (**2**–**4**), three phenylethanoid glycosides (**5**–**7**), four lignans (**8**–**11**) and three phenolic compounds (**12**–**14**) were isolated from the EtOH extract of Leonuri Fructus. Their structures were elucidated on the basis of 1D NMR, 2D NMR and HR-ESI-MS data analysis. All isolates were tested for their antioxidant, tyrosinase and acetylcholinesterase inhibitory activities. Most of them showed moderate antioxidant activities. Compounds **2**–**4** exhibited obvious inhibitory activities against mushroom tyrosinase at 25 $\mu$M, with %inhibition values of 49.36 ±2.69%, 43.43 ± 3.35%, 51.69 ± 2.81%, respectively, with arbutin used as the positive control (51.90 ± 2.57%). Compounds **3**, **5**–**6** and **9**–**10** exhibited significant inhibitory activities against acetylcholinesterase, similar to the positive control, galantamine.

**Keywords:** Leonuri Fructus; *Leonurus japonicus*; nortriterpenoid; phenylethanoid glycoside; tyrosinase; acetylcholinesterase. 

## 1. Introduction

*Leonurus japonicus* Houtt. is an herbaceous flowering plant native to Asia, and its crude extract has been reported to show cardioprotective, antioxidative, anticancer, neuroprotective, analgesic, anti-inflammatory, anthelminthic and antibacterial activities [1]. The air-dried ripe fruit of *Leonurus japonicus* Houtt, also called Leonuri Fructus, is recorded in the Pharmacopoeia of the People's Republic of China as a commonly used traditional Chinese medicine, with the effects of promoting blood circulation and regulating menstruation, clearing liver and improving eyesight [2]. So far, cyclic peptides, triterpenes, flavones and sterols have been isolated from Leonuri Fructus, which have many pharmacological effects such as antioxidation, neuroprotection, anti-inflammatory and antivirus. During the search for natural tyrosinase inhibitors from traditional Chinese medicines [3–5], the

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ethanolic extract of the Leonuri Fructus attracted our attention for its obvious inhibition activity against tyrosinase. A further investigation led to the isolation of fourteen secondary metabolites (Figure 1), three of which showed strong tyrosinase inhibitory activities, and five of the isolates exhibited obvious acetylcholinesterase inhibitory activities. Herein, the purification, characterization and bioactivities evaluation of these compounds were described.

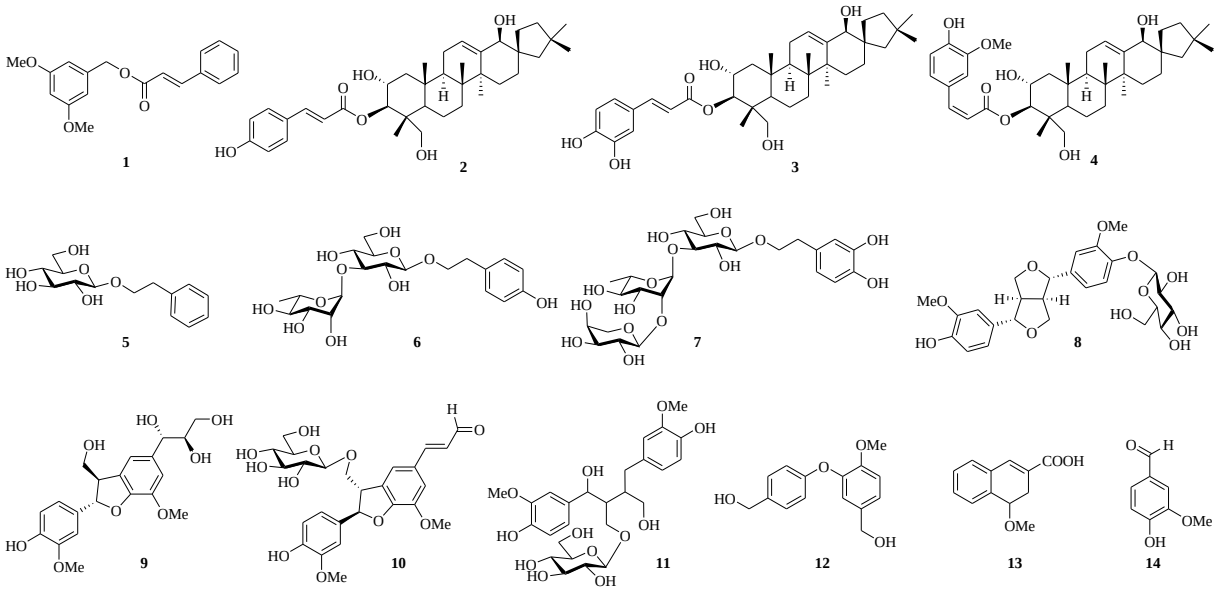


**Figure 1**. Chemical structures of compounds **1**–**14**

## 2. Materials and Methods

### *2.1. General Experimental Procedures*

Column chromatography (CC) was performed using silica gel (Qingdao Marine Chemical Inc., China), RP-$C_{18}$ (50 $\mu$m, Fuji Silysia Chemical Ltd., Japan) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). TLC was conducted with silica gel 60 F254 (Yantai Chemical Industry Research Institute). The spots on TLC were visualized by warming 10% $H_2SO_4$ (10% $H_2SO_4$ in ethanol) sprayed plates on a hot plate. $^1$D and 2D NMR spectra were collected by a Bruker AM-400 spectrometer (400 MHz). UV and FT-IR spectra were determined using Puxi TU-1950 and FTIR-650 instruments, respectively. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were carried out on a Bruker micrOTOF II spectrometer.

### *2.2. Plant Material*

The air-dried Leonuri Fructus was collected in Yuzhou, P. R. China, in October 2020 and authenticated by Prof. Lin Yang at Lanzhou University of Technology. The specimen (SPH2020D) was stored in Food and Pharmacy College, Xuchang University.

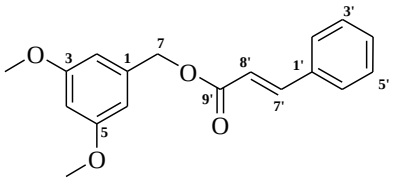
### *2.3. Extraction and Isolation*

The air-dried Leonuri Fructus (2.0 kg) was powdered and extracted with 95% EtOH (3 × 10 L, 50 °C). The solvent was removed at reduced pressure to yield a dark crude extract (50.6 g), which was suspended in $H_2O$ and extracted by $CH_2Cl_2$, EtOAc and *n*-BuOH. The EtOAc fraction (10.2 g) was subjected to silica gel CC eluting with gradients of petroleum ether–EtOAc–MeOH (20:1:0 to 0:0:1) to give ten fractions F1–F10. The fraction F2 (0.4 g, eluted by petroleum ether–EtOAc 15:1) was purified

by CC on silica gel (petroleum ether–EtOAc 20:1 to 10:1) to give compounds **1** (30.5 mg) and **14** (11.7 mg). The fraction F4 (0.3 g, eluted by petroleum ether–EtOAc 10:1) was purified by on RP-$C_{18}$ CC (MeOH–$H_2O$, 40:60 to 100:0) to afford compounds **12** (4.9 mg) and **13** (9.3 mg). The fraction F7 (2.1 g, eluted by petroleum ether–EtOAc 3:1) was further separated using Sephadex LH-20 column (MeOH) to give five subfractions (F7-1 to F7-5). Compounds **8** (12.3 mg) and **10** (10.9 mg) were gained from subfraction F7-1 by RP-$C_{18}$ CC using MeOH-$H_2O$ (20:80 to 100:0) as eluents. Compounds **5** (6.1 mg) and **9** (7.7 mg) were obtained from subfraction F7-4 by Sephadex LH-20 column (MeOH). The fraction F8 (0.9 g, eluted by petroleum ether–EtOAc 1:1) was passed through a RP-$C_{18}$ CC using MeOH-$H_2O$ (15:85 to 100:0) as eluents to give six subfractions (F8-1 to F8-6). The subfraction F8-1 was purified on the silica gel CC to give compounds **7** (13.8 mg) and **11** (7.3 mg). The subfraction F8-2 was chromatographed by Sephadex LH-20 column (MeOH) to afford compound **3** (9.5 mg). Compound **2** (8.6 mg) was obtained from subfraction F8-3 by recrystallization with MeOH. After further purification with Sephadex LH-20 column (MeOH), the fraction F9 gave compounds **4** (7.1 mg) and **6** (6.8 mg).

*Compound **1***. Colorless oil. IR (KBr) $\nu_{max}$ 2956, 2933, 2836, 1718, 1639, 1602, 1455, 1309, 1253, 1157, 1066, 977, 838, 767 $cm^{-1}$. UV $\lambda_{max}$ ($CHCl_3$) nm (log $\varepsilon$): 280 (3.5). HR-ESI-MS *m/z* 299.1282 $[M + H]^+$ (calcd for $C_{18}H_{19}O_4$, 299.1283). $^1$H-NMR ($CDCl_3$, 400 MHz) and $^{13}$C-NMR ($CDCl_3$, 100MHz) see Table 1.

**Table 1.** $^1$H and $^{13}$C NMR data of **1**[a]




| Position | $\delta_C$ | $\delta_H$ (mult, *J*, Hz) | position | $\delta_C$ | $\delta_H$ (mult, *J*, Hz) |
|---|---|---|---|---|---|
| 1 | 138.4 | | 3′ | 128.3 | 7.52 (dd, 6.4, 6.4) |
| 2 | 106.1 | 6.56 (d, 2.4) | 4′ | 130.5 | 7.36 (m) |
| 3 | 161.1 | | 5′ | 128.3 | 7.52 (dd, 6.4, 6.4) |
| 4 | 100.3 | 6.43 (t, 2.4) | 6′ | 129.0 | 6.49 (dd, 6.4, 2.8) |
| 5 | 161.1 | | 7′ | 145.4 | 7.73 (d, 16.0) |
| 6 | 106.1 | 6.56 (d, 2.4) | 8′ | 117.9 | 6.49 (d, 16.0) |
| 7 | 66.4 | 5.18 (s) | 9′ | 166.8 | |
| 1′ | 134.5 | | 3-OMe | 55.5 | 3.79 (s) |
| 2′ | 129.0 | 6.49 (dd, 6.4, 2.8) | 5-OMe | 55.5 | 3.79 (s) |

[a]400 MHz for $^1$H and 100 MHz for $^{13}$C, recorded in $CDCl_3$.

*2.4. DPPH Radical Scavenging Assay*

DPPH radical scavenging assay was conducted according to literature procedures [6], using L-ascorbic acid as the positive control. All tested compounds were dissolved in DMSO. Briefly, a mixture containing 20 $\mu$L of a sample solution (1 mM) and 180 $\mu$L of DPPH methanol solution (0.2 mM) were added to 96-well microplate, which was then incubated for 30 min in the dark. Antioxidant activities were determined by measuring absorbances at 517 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific, Inc.). Experiments were performed in triplicate. The DPPH radical scavenging activity was calculated by: Radical scavenging activity (%) =(Ac − As)/Ac × 100, where Ac is the absorbance of the non-treated control and As is the absorbance of tested compound.

*2.5. Acetylcholinesterase Inhibition Assay*

Acetylcholinesterase inhibition assay was conducted according to the literature procedures with slight modification [7]. Compounds **1**–**14** were dissolved in DMSO. A mixture containing 140 $\mu$L phosphate buffer (pH 8.0), 20 $\mu$L of the compound (1 mg/mL), 20 $\mu$L of AChE solution (0.4U/ml) and 20 $\mu$L of DTNB was added in a 96-well microplate and incubated for 15 min at 25 °C. The reaction was then initiated with the addition of 10 $\mu$L of acetylthiocholine iodide. Hydrolysis of acetylthiocholine iodide was monitored by the formation of the yellow 5-thio-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at 412 nm utilizing a 96-well microplate reader. Galantamine was used as the positive control. All reactions were repeated three times. The %inhibition was calculated as follows: %inhibition = (1 − S/E) × 100, where E is the activity of enzyme without test sample and S is the activity of the enzyme with test sample. The results were analyzed by program GraphPad Prism 5.0. Data are expressed as means ± SEMs of triplicate.

*2.6. Mushroom Tyrosinase Inhibition Assay*

Mushroom tyrosinase inhibition assay was performed according to the literature procedures [8], using arbutin as the positive control. All experiments were repeated three times. The %inhibition was calculated by (Ac − As)/Ac × 100, where As is the absorbance of tested compound and Ac the non-treated control. The results were analyzed by program GraphPad Prism 5.0. Data are expressed as means ± SEMs of triplicate.

## 3. Results and Discussion

*3.1. Isolation and Structure Elucidation*

The air-dried Leonuri Fructus was extracted with 95% EtOH to afford the EtOH extract, which was sequentially fractionated into $CH_2Cl_2$-, EtOAc-, *n*-BuOH- and $H_2O$-soluble fractions. The tyrosinase and acetylcholinesterase inhibitory activities were valuated among all crude extracts at a concentration of 100 $\mu$g/mL. As shown in Table 2, the EtOAc-soluble fraction showed the strongest inhibitory activities against both tyrosinase and acetylcholinesterase. Therefore, the EtOAc-soluble fraction was chosen for further investigation on its chemical constituents in this study.

**Table 2.** Inhibitory activities of various fractions obtained from Leonuri Fructus on tyrosinase and acetylcholinesterase[a]

| Fraction | Tyrosinase inhibition (%) | Acetylcholinesterase inhibition (%) |
|---|---|---|
| Control (100% DMSO) | NI[b] | NI[b] |
| 95% EtOH extract | 15.73±1.09 | 23.49±0.88 |
| $CH_2Cl_2$-Soluble fraction | 11.69±1.64 | 15.66±0.95 |
| EtOAc-Soluble fraction | 27.62±1.01 | 36.59±0.60 |
| *n*-BuOH-Soluble fraction | 13.71±1.50 | 9.53±1.11 |
| $H_2O$-Soluble fraction | 9.88±1.12 | 13.36±0.89 |
| arbutin[c] | 48.99±1.27 | |
| galantamine[d] | | 83.23±0.72 |

[a]Measured at 100 $\mu$g/mL, results were shown as means ±SEMs. [b]NI: no inhibition. [c] Positive control, 25 $\mu$M. [d]Positive control, 50 $\mu$M.

After repeated column chromatography over silica gel, RP-C18 and Sephadex LH-20, as well as recrystallization, the EtOAc-soluble fraction gave fourteen compounds (**1**–**14**), one of which was a new compound. The thirteen known ones (**2**–**14**) were identified as leonurusoleanolide A (**2**) [9], leonurusoleanolide J (**3**) [10], leonurusoleanolide D (**4**) [9], 2-phenylethyl *β*-D-glucopyranoside (**5**) [11], cistanoside G (**6**) [12], leonuriside B (**7**) [13], (+)-pinoresinol *O*-*β*-D-glucopyranoside (**8**) [14], meliasendanin B (**9**) [15], tortoside F (**10**) [16], cinnacassoside A (**11**) [17], 4,5'-bishydroxymethyl-2'-methoxydiphenylether (**12**) [18], eriosematin F (**13**) [19], 4-hydroxy-3-methoxybenzaldehyde (**14**) [20], respectively, by spectral comparison with literature. Compounds **1** and **5**–**13** were reported from Leonuri Fructus for the first time, although compounds **7** and **14** have been isolated from Leonurus japonicus by Sugaya et al [21] and Zhou et al [22], respectively. The structure of the new compound (**1**) was identified based on extensive spectroscopic analysis as described below.

Compound **1** was obtained as a colorless oil. The molecular formula $C_{18}H_{18}O_4$, with ten degrees of unsaturations, was established based on its quasi-molecular ion peak at *m/z* 299.1282 $[M + H]^+$ (calcd for $C_{18}H_{19}O_4$, 299.1283) in the HR-ESI-MS spectrum. The IR absorption band at 1718 $cm^{-1}$ suggested the presence of a carbonyl group. The $^1$H-NMR spectrum of **1** revealed the signals of an $A_2B$ spin system at $\delta_H$ 6.56 (2H, d, *J* = 2.4 Hz, H-2, H-6), 6.43 (1H, t, *J* = 2.4 Hz, H-4), suggesting the presence of one 1, 3, 5-tirsubstituted benzene ring. Additionally, a typical coumaroyl moiety was observed, which included a *trans*-double bond [$\delta_H$ 7.73 and 6.49 (each 1H, d, *J* = 16.0 Hz, H-7′ and H-8′)], a benzene ring with an $A_2B_2X$ system [$\delta_H$ 7.52 (2H, dd, *J* = 6.4, 6.4 Hz, H-3′, 5′), 7.36 (1H, m, H-4′), 6.49 (2H, dd, *J* = 6.4, 2.8 Hz, H-2′, 6′)]. Apart from fifteen typical carbon signals assigned for the 1, 3, 5-tirsubstituted benzene ring [$\delta_C$ 161.1 (C-3, C-5), 138.4 (C-1), 106.1 (C-2, C-6), 100.3 (C-4)], and coumaroyl moiety [$\delta_C$ 166.8 (C-9′), 145.4 (C-7′), 134.5 (C-1′), 130.5 (C-4′), 129.0 (C-2′, C-6′), 128.3 (C-3′, C-5′), 117.9 (C-8′)], the $^{13}$C and DEPT NMR spectroscopic data of **1** exhibited only three resonances attributable to two methoxy groups [$\delta_C$ 55.5 (3-O*C*H$_3$, 5-O*C*H$_3$) and one methylene [$\delta_C$ 66.4 (C-7)]. The speculations above were confirmed by the key $^1$H-$^1$H COSY and HMBC correlations shown in Figure 2. The HMBC correlations from H-7 to C-9′ showed that the connection between 1, 3, 5-tirsubstituted benzene ring and coumaroyl moiety occurred at C-7 position. The strong cross-peaks from 3-O*CH*$_3$ to C-3, from 5-O*CH*$_3$ to C-5 indicated that the two methoxy groups were attached to C-3, C-5, respectively. Therefore, the structure of compound **1** was identified as 3,5-dimethoxybenzyl cinnamate.

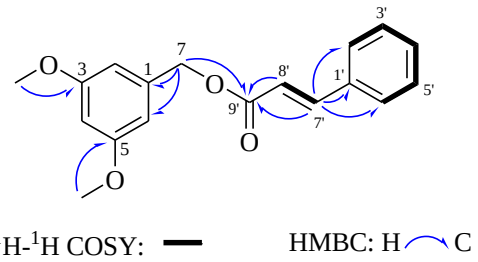



**Figure 2**. Key $^1$H-$^1$H COSY and HMBC correlations for compound **1**

*3.2. Antioxidant, tyrosinase and acetylcholinesterase inhibitory activities*

Compounds **1**–**14** were evaluated for their antioxidant activities with DPPH radical scavenging assay. As shown in Table 3, compounds **2** and **4** exhibited obvious DPPH radical scavenging activities: 57.73±1.59% for **2**; 54.37±1.94% for **4**; 61.41±2.74% for L-ascorbic acid (positive control). The DPPH radical scavenging activities of **2** and **4** might be ascribed to the hydroxyl substituents at their benzene rings as well as spirocyclic nortriterpenoid moieties. Besides, phenylethanoid glycosides **5**, **6**, lignans **9**–**11** and phenolic compound **13** also showed moderate antioxidant activities.

**Table 3.** DPPH radical scavenging activity of compounds **2–7**, **9–11**, **13–14** and L-ascorbic acid[a]

| Compound | DPPH radical scavenging activity (%) | Compound | DPPH radical scavenging activity (%) |
|---|---|---|---|
| **2** | 57.73±1.59 | **9** | 36.96±2.13 |
| **3** | 21.59±2.62 | **10** | 25.84±2.45 |
| **4** | 54.37±1.94 | **11** | 37.53±2.10 |
| **5** | 31.07±2.56 | **13** | 20.44±2.71 |
| **6** | 34.51±2.33 | **14** | 23.30±4.12 |
| **7** | 27.88±2.53 | L-ascorbic acid (positive control) | 61.41±2.74 |

[a]Measured at a concentration of 1 mM. Three independent experiments were performed and results were expressed as means ± SEMs.

At a concentration of 25 $\mu$M, using arbutin as the positive control, all isolated compounds were evaluated for their tyrosinase inhibitory activities. However, only the three spirocyclic nortriterpenoids **2**–**4** showed obvious inhibitory activities, with %inhibition values of 49.36±2.69%, 43.43±3.35%, 51.69±2.81%, respectively, very close to the positive control arbutin (51.90±2.57%) (Table 4). Further experiments demonstrated that the $IC_{50}$ values of **2**–**4** were also similar to that of arbutin (35.25±0.11 $\mu$M for **2**, 36.75±0.09 $\mu$M for **3**, 38.72±0.13 $\mu$M for **4**, 30.18±0.12 $\mu$M for arbutin). Compounds **2**–**4** contain a unique 28-noroleanane-type spirocyclic skeleton. This type of compounds has been reported from many plants in the Lamiaceae family, such as *Gomphostemma parviflorum* [23], *Notochaete hamosa* [24] and *Phlomis viscosa* [25], and have been reported to show potent cytotoxic activities [26-27] and NGF-potentiating activity [9]. Interestingly, so far inhibitory activities against tyrosinase have not been found for 28-noroleanane-type spirocyclic triterpenoids. As the first report about their tyrosinase inhibitory activities, these results would be of great instruction to novel tyrosinase inhibitors development and medicinal value utilization of Leonuri Fructus.

**Table 4.** Inhibitory effects of compounds **2–4** and arbutin on mushroom tyrosinase[a]

| Compound | Tyrosinase inhibition (%) | $IC_{50}$ ($\mu$M) |
|---|---|---|
| **2** | 49.36±2.69 | 35.25±0.11 |
| **3** | 43.43±3.35 | 36.75±0.09 |
| **4** | 51.69±2.81 | 38.72±0.13 |
| arbutin (positive control) | 51.90±2.57 | 30.18±0.12 |

[a]Measured at 25 $\mu$M. Results were expressed as means ± SEMs.

The acetylcholinesterase inhibitory effects of compounds **1**−**14** were evaluated, and the results were shown in Table 5. At a concentration of 50 $\mu$M, compounds **3**, **5**–**6**, **9–10** showed strong inhibitory activity (>55%) against acetylcholinesterase, with %inhibition values of 55.23±0.77%, 77.79±0.47%, 70.29±1.21%, 70.89±2.05%, 77.02±1.51%, respectively. Compounds **2** and **4** showed weak inhibitory activity, indicating that the coumaroyl moiety attached to the C-3 position might influence the acetylcholinesterase inhibitory activities of nortriterpenoids. Lignins with a benzofuran skeleton (**9**, **10**) showed stronger inhibitory activities than others (**8**, **11**).

**Table 5.** Acetylcholinesterase inhibitory effects of compounds **3**, **5**, **6**, **9**–**10** and galantamine[a]

| Compound | Acetylcholinesterase inhibition (%) |
|---|---|
| **3** | 55.23±0.77 |
| **5** | 77.79±0.47 |
| **6** | 70.29±1.21 |
| **9** | 70.89±2.05 |
| **10** | 77.02±1.51 |
| galantamine (positive control) | 88.85±0.69 |

[a]Measured at 50 $\mu$M. Three independent experiments were performed and results were expressed as means ± SEMs.

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## Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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