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# A new flavonol glycoside and activity of compounds from the flower of *Nymphaea candida*

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A new compound, kaempferol 3-*O*-(2″-*O*-galloylrutinoside) (**1**), was isolated from the white flower of *Nymphaea candida*, together with nine known flavonol glycosides, kaempferol (**2**), kaempferol 3-*O*-β-D-glucopyranoside (**3**), kaempferol 3-*O*-α-L-rhamnopyranoside (**4**), kaempferol 3-*O*-α-L-rhamnopyranosylglucopyranoside (**5**), kaempferol 7-*O*-β-D-glucopyranoside 3-(*O*-α-L-rhamnopyranosylglucopyranoside) (**6**), quercetin (**7**), quercetin 3-*O*-β-D-xylopyranoside (**8**), myricetin (**9**), myricetin 3′-*O*-β-D-xylopyranoside (**10**). The structure of **1** was established on the basis of the analysis of its 1D and 2D NMR spectral data. Compounds **1**–**7** and **9** exhibited moderate to significant antioxidant activities, which were evaluated by measurement of low-density lipoprotein (LDL) and malondialdehyde (MDA) levels in vitro. Compounds **1**, **3**, **4**, **6** and **9** exhibited promising neuroprotective effects on ischemic injury model of cultured rat cortical neurons treated with sodium dithionite in glucose-free medium. Furthermore, compounds **1**, **5**, and **9** had distinct cytotoxicity to adrenal gland pheochromocytoma, PC12 cells, being treated by the same way.

*Keywords*: Flavonoid; Kaempferol 3-*O*-(2″-*O*-galloylrutinoside); Cytotoxicity; Neuroprotection; Antioxidation; *Nymphaea candida*

## 1. Introduction

*Nymphaea candida* Presl., which belongs to the family Nymphaeaceae, is mainly distributed in Siberia, the middle part of Asia and Europe as an ornamental and a foodstuff [1]. In China, this plant is cultivated in Xinjiang and its flower has been used for a traditional Chinese medicine, with the function of clearing inner heat and toxin, calming the mind and sedation, for the treatment of common cold, fever, headache, cough, and faucitis [2]. Previous chemical studies carried out on the flower and leaf of *N. candida* have resulted in the isolation of several anthocyanins such as delphinidin-3-galactoside, cyaniding-3-galactoside, delphinidin-7-galactoside, and two unknown alkanoids [3,4]. However, no systemic investigations of the chemical constituents and bioactivity on the flower of *Nymphaea candida* have been carried out as yet.

The present paper deals with the isolation and characterisation of a new compound, kaempferol 3-*O*-(2″-*O*-galloylrutinoside) (**1**), from the ethanol extract of the dried white

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flower of *N. candida*, and investigates the neuroprotective, cytotoxic and antioxidant activities of this new compound, together with nine known flavonol glycosides, kaempferol (**2**), kaempferol 3-*O*-β-D-glucopyranoside (**3**), kaempferol 3-*O*-α-L-rhamnopyranoside (**4**), kaempferol 3-*O*-α-L-rhamnopyranosylglucopyranoside (**5**), kaempferol 7-*O*-β-D-glucopyranoside 3-(*O*-α-L-rhamnopyranosylglucopyranoside) (**6**), quercetin (**7**), quercetin 3-*O*-β-D-xylopyranoside (**8**), myricetin (**9**), myricetin 3′-*O*-β-D-xylopyranoside (**10**).

## 2. Results and discussion

Compound **1** was obtained as a pale yellow needle-like crystal with a melting point of 238–240°C. Its molecular formula of $C_{34}H_{34}O_{19}$ was determined by HRESI-MS and indicated 18 degrees of unsaturation. The UV spectrum of **1** exhibited typical flavonol absorptions at 267 and 347 nm. The $^1$H NMR spectrum of **1** showed an AA′BB′ system at $\delta$ 7.94 (2H, d, $J = 8.3$ Hz, H-2′,6′) and 6.88 (2H, d, $J = 8.3$ Hz, H-3′,5′), and an AX system at $\delta$ 6.38 (1H, d, $J = 1.5$ Hz, H-8) and 6.17 (1H, d, $J = 1.5$ Hz, H-6), in accordance with kaempferol derivatives. The two anomeric signals at $\delta$ 5.62 (1H, d, $J = 8.0$ Hz) and 4.36 (1H, br s) indicated the presence of two sugar units with β- and α-configurations, respectively. The doublet at $\delta$0.98 (3H, d, $J = 6.5$ Hz) was a particular signal of 6-methyl group in rhamnopyranosyl residue. The singlet at $\delta$7.05 (2H, s, galloyl H-2″,6″), which was correlated to a carbon at $\delta$ 108.9 (galloyl C-2″,6″) in the HMBC spectrum, was in agreement with gallic acid (3,4,5-trihydrobenzoic acid) [5,6]. The $^1$H NMR and $^{13}$C NMR data of **1** were similar to those of quercetin 3-*O*-(2″-*O*-galloylrutinoside) [7], but with default of 3′-hydroxy signals in **1** (see table 1). These suggested that **1** should be kaempferol 3-*O*-(2″-*O*-galloylrutinoside). This was further confirmed by HMQC and HMBC correlations (see figure 1). HMBC correlations between H-1″ ($\delta$ 5.62) of glucose and C-3 ($\delta$ 132.5) of aglycone, H-2″ ($\delta$ 4.90) of glucose and galloyl carbonyl ($\delta$ 165.1), H-1″ ($\delta$ 4.36) of rhamnose and C-6″ ($\delta$ 67.0) of glucose, indicated that the glucose should be attached to C-3 of aglycone, the galloyl group was connected to the C-2″ of glucose and the rhamnose was located at the C-6″ position of glucose, specifically. The overall structural determination of **1** was based on the detailed analysis of 1D and 2D NMR data including the $^1$H NMR, $^{13}$C NMR, HMQC, and HMBC spectra. On the basis of above evidences, compound **1** was assigned as 3-[6-*O*-(6-deoxy-α-L-mannopyranoxyl)-2-*O*-(3,4,5-trihydroxybenzoyl)-β-D-glucopyranosyl]oxy-2-(4-hydroxyphenyl)-5,7-dihydroxy-4H-1-benzopyran-4-one, or kaempferol 3-*O*-(2″-*O*-galloylrutinoside).

Nine known compounds were identified by comparison of their spectrometric data with reported values as kaempferol (**2**) [8], kaempferol 3-*O*-β-D-glucopyranoside (**3**) [8], kaempferol 3-*O*-α-L-rhamnopyranoside (**4**) [9], kaempferol 3-*O*-α-L-rhamnopyranosylglucopyranoside (**5**) [8], kaempferol 7-*O*-β-D-glucopyranoside 3-(*O*-α-L-rhamnopyranosylglucopyranoside) (**6**) [10], quercetin (**7**) [6], quercetin 3-*O*-β-D-xylopyranoside (**8**) [11], myricetin (**9**) [12], myricetin 3′-*O*-β-D-xylopyranoside (**10**) [6].

To investigate the bioactivities of flavonol glycosides **1**–**10** on ischemic injury in cultured rat cortical neurons and the rat pheochromocytoma PC12 cells, cortical neurons of rat and PC12 cells were cultured *in vitro*. The effects of compounds on ischemic injury by treating cells with sodium dithionite in glucose-free medium were observed. Compounds **1**, **3**, **4**, **6** and **9** reduced cell death and decreased medium lactic dehydrogenase (LDH) levels. However, compounds **1**, **5**, and **9** strongly increased LDH leakage from the PC12 cells, and showed cell death which was evident upon microscopic examination (see table 2).

Table 1. $^{1}$H NMR and $^{13}$C NMR data of **1** in DMSO (500 MHz for $^{1}$H and 125 MHz for $^{13}$C, $\delta$ in ppm, $J$ in Hz).

| *No.* | *$^{1}$H* | *$^{13}$C* | *HMBC ($^{1}$H to $^{13}$C)* |
|---|---|---|---|
| 2 | | 156.4 | |
| 3 | | 132.5 | |
| 4 | | 176.9 | |
| 5 | | 161.1 | |
| 5-OH | 12.5 (1H, s) | | C-5, 6, 10 |
| 6 | 6.17 (1H, d, $J = 1.5$) | 98.6 | C-5, 7, 8, 10 |
| 7 | | 164.0 | |
| 8 | 6.38 (1H, d, $J = 1.5$) | 93.7 | C-6, 7, 9, 10 |
| 9 | | 159.9 | |
| 10 | | 104.0 | |
| 1′ | | 120.7 | |
| 2′ (6′) | 7.94 (2H, d, $J = 8.5$) | 130.8 | C-2, 2′ (6′), 4′ |
| 3′ (5′) | 6.88 (2H, d, $J = 10.5$) | 115.1 | C-2, 1′, 3′ (5′) |
| 4′ | | 156.9 | |
| 3-*O*-β-glucopyranoside | | | |
| 1″ | 5.62 (1H, d, $J = 8.5$) | 98.6 | C-3 |
| 2″ | 4.90 (1H, t, $J = 8.0$) | 74.1 | C-carbonyl, 1″ |
| 3″ | | 75.8 | |
| 4″ | | 70.3 | |
| 5″ | | 74.0 | |
| 6″ | | 67.0 | |
| 2″-galloyl carbonyl | | 165.1 | |
| 1″ | | 119.6 | |
| 2″ (6″) | 7.05 (2H, s) | 108.9 | C-1″, 2″ (6″), 3″ (5″), 4″ |
| 3″ (5″) | | 145.4 | |
| 4″ | | 138.3 | |
| 6″-deoxy-α-mannopyranosyl | | | |
| 1″ | 4.36 (1H, br s) | 100.9 | C-6″, 2″ |
| 2″ | | 70.3 | |
| 3″ | | 70.6 | |
| 4″ | | 71.7 | |
| 5″ | | 68.3 | |
| 6″ | 0.98 (3H, d, $J = 6.0$) | 17.7 | C-4″, 5″ |

The antioxidant activities of compounds **1**–**10** were estimated by measurement of low-density lipoprotein (LDL) susceptibility to oxidation, and determination of malondialdehyde (MDA) levels in the rat brain *in vitro*. The data collected (see table 3) indicated that both new compound **1** and known compounds **2**–**7** and **9** exhibited moderate to significant antioxidant bioactivity.

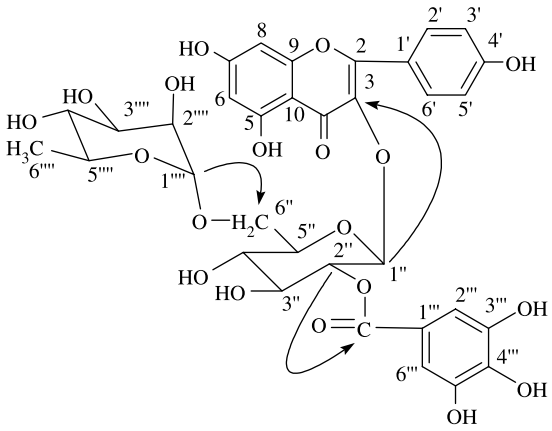


Figure 1. Key HMBC correlations of **1**.

Table 2. Effect of compounds **1**–**10** on cultured rat cortical neurons and PC12 cells treated with sodium dithionite in glucose-free medium *in vitro* [†].

| *Agents* | *PC12 cells' LDH leakage (%) ± SD*[‡] | *Rat cortical neurons' LDH leakage (%) ± SD*[‡] |
|---|---|---|
| Normal | 17.5 ± 0.02 | 21.1 ± 0.02 |
| Control | 35.3 ± 0.02 | 50.4 ± 0.01 |
| **1** | 88.9 ± 0.07** | 22.8 ± 0.02** |
| **2** | 28.6 ± 0.04* | 60.1 ± 0.04 |
| **3** | 31.7 ± 0.04 | 35.9 ± 0.05** |
| **4** | 38.7 ± 0.04 | 24.8 ± 0.04** |
| **5** | 84.6 ± 0.02** | 47.8 ± 0.02 |
| **6** | 35.8 ± 0.02 | 26.8 ± 0.05** |
| **7** | 41.3 ± 0.03* | 56.6 ± 0.04 |
| **8** | 45.6 ± 0.06* | 53.0 ± 0.05 |
| **9** | 91.9 ± 0.02** | 33.9 ± 0.10** |
| **10** | 62.0 ± 0.10* | 66.6 ± 0.03** |

[†] For the protocols used, see Section 3.
[‡] Values are the mean of lactic dehydrogenase (LDH) leakage percent ± SD ($n = 3$, $*P < 0.05$, $**P < 0.01$, versus control), calculated by the formula: LDH activity$_{\text{extra-cell}}$/(LDH activity$_{\text{extra-cell}}$ + LDH activity$_{\text{intra-cell}}$) × 100%, final concentrations were $1 \times 10^{-5}$ g ml$^{-1}$.

The antioxidant and cytotoxic activities reported herein for the flower of *N. candida* are supported by the clinical uses of this Chinese traditional medicine. However, the neuroprotective activities are not found in its folk use, which may lead to the better utilisation of *N. candida* flower in the future.

## 3. Experimental

### *3.1 General experimental procedures*

Melting point was determined on an XT–4A micromelting point apparatus and is uncorrected. UV spectra were run on a Varian Cary Eclipse 300 spectrometer using methanol as solvent. $^{1}$H NMR and $^{13}$C NMR experiments were performed on a Bruker DRX 500 NMR spectrometer using DMSO-$d_6$ as internal standard. The HRESI-MS were run with a Bruker APEX II mass spectrometer. Column chromatography was carried out with polyamide (30–60, 60–90 mesh)

Table 3. Effects of compounds **1**–**10** on conjugated diene (CD) formation of LDL or malondialdehyde (MDA) in rat brain *in vitro* [†].

| *Agents* | *Concentration* ($1 \times 10^{-5}$ *g ml*$^{-1}$) | *Percentage increase of CD(%) ± SD*[‡] | *Inhibition rate of MDA (%)*[‡,¶] |
|---|---|---|---|
| Control | | 55.33 ± 4.12 | |
| Vitamin C | 3 | 0.12 ± 0.86** | 100** |
| **1** | 3 | 19.67 ± 4.92** | 140.3** |
| **2** | 3 | 33.49 ± 4.33* | 153.9** |
| **3** | 3 | 6.35 ± 1.02** | 91.3** |
| **4** | 3 | 15.00 ± 1.24** | 101.5** |
| **5** | 3 | 20.16 ± 1.24** | 83** |
| **6** | 3 | 25.83 ± 3.10** | 73.5** |
| **7** | 3 | 46.31 ± 5.28 | 159.8** |
| **8** | 3 | 60.59 ± 9.10 | 31.4* |
| **9** | 3 | 37.99 ± 3.60* | 106.6** |
| **10** | 3 | 50.94 ± 1.06 | 106.7** |

[†] For protocols used, see the Experimental Section of Ref. [15].
[‡] $n = 3$, $*P < 0.05$, $**P < 0.01$, versus control.
[¶] The inhibition rate was evaluated by the formula (MDA$_{\text{control}}$ − MDA$_{\text{agent}}$)/(MDA$_{\text{control}}$ − MDA$_{\text{Vc}}$) × 100%, $n = 2$.

(Jiangsu Linjiang Reagent Chemistry Co. Ltd.), silica gel (100–300 mesh) (Tsingtao Marine Chemistry Co. Ltd.), Sephadex LH–20 (18–110 μm) (Pharmacia Co. Ltd.), and ODS (100–200 mesh) (Fuji Silysia Chemical Co. Ltd.). Laboratory animals were obtained from the Laboratory Animal Institute, Chinese Academy of Medical Science, Beijing, China.

### *3.2 Plant material*

The plant material was collected in September 2002 from Aletai City, Xinjiang Province, China, and identified by Dr. Wei Wang as the flower of *Nymphaea candida* Presl., and a voucher specimen (No. 030708) has been deposited in the herbarium of the Laboratory of Pharmaceutical Science, Department of Biological Sciences and Biotechnology, Tsinghua University.

### *3.3 Extraction and isolation*

The dried flowers (7 kg) were extracted with 95% ethanol under reflux. After concentration *in vacuo*, the ethanol extract (980 g) was suspended in water and partitioned successively with petroleum ether, ethyl acetate, and *n*-butanol. The ethyl acetate-soluble part (360 g) was subjected to polyamide column chromatography using water/ethanol mixtures (10:0 → 2:8), 95% ethanol as eluents to afford 100 fractions (Fr1–Fr100). Fractions 35–38 were chromatographed on a silica gel column sequentially eluted with $CHCl_3$/MeOH (C—M) mixtures (95:5 → 50:50) to give compounds **1** (180 mg, C—M 60:40), **3** and **4** mixture (C—M 8:2). The latter was chromatographed on a Sephadex LH-20 column with 70% methanol, and received compounds **3** (35 mg) and **4** (10 mg). Fractions 26–34 were subjected to polyamide column chromatography using 30% ethanol as eluent to give compound **5** (60 mg). Fractions 45–46 and fractions 65–72 were treated separately subjected to silica gel column chromatography with a gradient C–M mixtures and purified by Sephadex LH-20 column chromatography with 70% methanol to yield compounds **8** (29 g) and **10** (9 mg); **2** (23 mg), **7** (16 mg) and **9** (8 mg). The *n*-butanol-soluble part (224 g) was subjected to a macroporous resin D101 column, using water and 20%, 50%, and 95% ethanol solution as eluents to provide four fractions. The 20% ethanol eluate was concentrated to dryness and further subjected to ODS column chromatography employing gradient MeOH/$H_2O$ mixtures (from 10% to 50%) as eluent to give fractions (A–E). fraction C was isolated and purified several times by ODS column chromatography using 40% ethanol as eluent to give compound **6** (10 mg).

### *3.4 Characterisation of the new compound*

Kaempferol 3-*O*-(2″-*O*-galloylrutinoside) **(1)**: pale yellow needle-like crystal (MeOH). Mp 238–240°C; $[\alpha]_D$ −2.96; UV (MeOH) $\lambda_{max}$ (log ε) 347 (4.12), 267 (4.36), 211 (4.58) nm; $^1$H NMR and $^{13}$C NMR, see table 1; HRESI-MS (negative) *m/z* 745.1617 $[M\text{-}1]^-$ (calcd for $C_{34}H_{33}O_{19}$, 745.1616).

### *3.5 Neuroprotective effect in primary rat cortical neurons and cytotoxicity to PC12 cells*

Primary cultures of rat cerebral cortex of 1-day-old newborn rats as described previously [13]. Neurons were plated at a density of $1 \times 10^5$ cells/well on poly-L-lysine-precoated

96-well plates. Cultures were maintained in modified Eagle's medium, 10% new born bovine serum, 5% glucose, 50 IU $ml^{-1}$ penicillin, and 0.05 mg $ml^{-1}$ streptomycin at 37°C with 95% air and 5% $CO_2$. After 2 days in culture, the cells were treated with 4 μg $ml^{-1}$ cytarabine to prevent proliferation of non-neuronal cells. Ischemic injury experiment caused by sodium dithionite was carried out after 6–7 days in culture. PC12 cells were placed in microtiter plated (96 wells) at a density of $1 \times 10^4$ cells/well. Cultures were maintained in the conditions supplied by ATCC. After 24 h the medium was carefully removed and cells were cultured in glucose-free Earle's medium (NaCl, 143 mM; KCl, 5.4 mM; $CaCl_2$, 1.8 mM; $Mg_2SO_4$, 1.0 mM; $NaH_2PO_4$, 1.0 mM; HEPES, 2.4 mM; pH 7.4) with sodium dithionite (0.5 mM) and different compounds(final concentrations were $1 \times 10^{-5}$ g $ml^{-1}$, prepared densely solutions dissolved in the sterile normal saline and filtered through 0.22 μm Millipore filter) after being washed with glucose-free Earle's medium twice. The model cell group was treated the same way without the compounds, and the control cell group was just supplemented with Earle's medium containing 10 mmol/L glucose [14]. After 5–6 h in culture, the medium was collected as an extra cellular solution. The intracellular solution was obtained through repeated refrigeration (− 20°C) and melting (4°C) of cells added with non-glucose Earle's solution. LDH activity was carried out by a method using a LDH kit (Beijing Zhongsheng biochemical reagent company, batch number: 040624).

### *3.6 Measurement of low-density lipoprotein (LDL) susceptibility to oxidation and determination of malondialdehyde (MDA) levels*

The measurement of LDL from the plasma of New Zealand White rabbits and the MDA levels in the brains of rats determined through the thiobarbituric acid (TBA) method were performed as described in the literature published previously [15].

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