SCIENTIFIC RESEARCH

Journal of Medicinal Materials, 2024, Vol. 29, No. 3 (pp. 131 - 139)

CONSTITUENTS ISOLATED FROM THE LEAVES OF *RINOREA BENGALENSIS* AND THEIR α-GLUCOSIDASE INHIBITORY EFFECTS

Kieu Thi Phuong Linh[1], Duong Thu Trang[1], Vu Thanh Trung[1], Pham Thanh Binh[1], Nguyen The Cuong[2], Nguyen Van Thanh[1], Nguyen Phuong Thao[1,*]

[1]*Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam;*

[2]*Institute of Ecology and Biological Resources, VAST, Cau Giay, Hanoi, Vietnam*

*Corresponding author: thaonp@imbc.vast.vn

(Received March 28th, 2024)

Summary

Constituents Isolated from the Leaves of *Rinorea bengalensis* and their α-Glucosidase Inhibitory Effects

Phytochemical investigation of the ethanolic extract of *Rinorea bengalensis* (Wall.) Kuntze leaves led to the isolation of five compounds (**1**–**5**), using various chromatographic separations. Their structures were elucidated to be 4-*C*-*β*-D-glucopyranosyl-3,5-dihydroxy-2-methoxybenzamide (**1**), bergenin (**2**), kaempferol 3-*O*-*β*-D-glucopyranosyl-(1'''→2'')-*O*-*α*-L-arabinopyranoside (**3**), (7*α*,8*α*)-dihydrodehyrodiconiferyl alcohol-9-*O*-*β*-D-glucopyranoside (**4**), and (+)-lariciresinol (**5**) by detailed analysis of 1D and 2D NMR and MS spectroscopic data as well as comparison with those reported. In the *in vitro* α-glucosidase inhibition assay, compounds **1**, **4**, and **5** showed inhibitory activity with IC_{50} values ranging from 11.57 ± 0.76 to 69.98 ± 1.36 μM. Enzyme kinetic analysis revealed that compound **1** behaved as a mixed-type inhibitor with a K_i value of 21.37 ± 0.08 μM. This is the first time that chemical constituents from *R. bengalensis* and their α-glucosidase inhibitory effect were reported.

Keywords: *Rinorea bengalensis*; *Violaceae*; *Phenolic*; *α-Glucosidase inhibitory effect.*

1. Introduction

Violaceae is a medium-sized cosmopolitan family belonging to Malpighiales with 26 accepted genera and approximately 1100 species of trees, shrubs, lianas, and herbs [1]. *Rinorea* is the genus of the family Violaceae and contains about 225-275 species distributed throughout tropical and subtropical areas of the world, including Africa, Asia, and Australia [2],[3]. Among them, *Rinorea bengalensis* (Wall.) Kuntze is a native Australian rainforest tree, but it is also located in Asia (Cambodia, China, India, Indonesia, Malaysia, Thailand, and Vietnam...), and the Pacific Islands [3],[4]. However, there is a lack of phytochemical and pharmacological data on this plant. Thus far, only two reports on the occurrence of cyclotide-like molecules have been performed to explore the chemical constituents [4],[5]. They have attracted attention for their cytotoxic properties and hemolytic activity [4]. Until now, no report regarding the α-glucosidase inhibition of *R. bengalensis* extracts and isolated compounds has appeared. Herein, the phytochemical investigation of the ethanolic extract of *R. bengalensis* leaves was performed and five phenolic compounds (**1**–**5**, Fig. 1) were isolated and elucidated by 1D and 2D NMR analysis. The α-glucosidase inhibitory activity of the phytochemicals from this plant is described. Additionally, to explain the underlying mechanism of the inhibitory activity of **1** on α-glucosidase, the enzyme kinetic analysis was also carried out.

2. Material and methods

2.1. Chemicals and equipment

The melting points were measured on a Thermo Scientific 1401 Mel-Temp 3.0 melting point apparatus. Optical rotations were determined on a JASCO P-2000 polarimeter. The UV spectra were recorded on a Chirascan spectrometer. A PerkinElmer Spectrum Two FT-IR spectropolarimeter was used to record the infrared spectra with KBr pellets. The NMR spectra were recorded on a Bruker AVANCE NEO 600 FT-NMR spectrometer with TMS as an internal standard. ESIMS was collected on an Agilent 6530 Accurate-Mass spectrometer. MPLC was carried out on a Biotage-Isolera One system. Column chromatography was conducted on *silica gel*, Diaion HP-20, Sephadex LH-20, and RP-18. Analytical thin-layer chromatography was performed on precoated *silica gel* 60 F_{254} and RP-18 F_{254S} plates and compounds were visualized by spraying with 10% H_2SO_4 in water and then heating for 1.5 - 2 minutes.

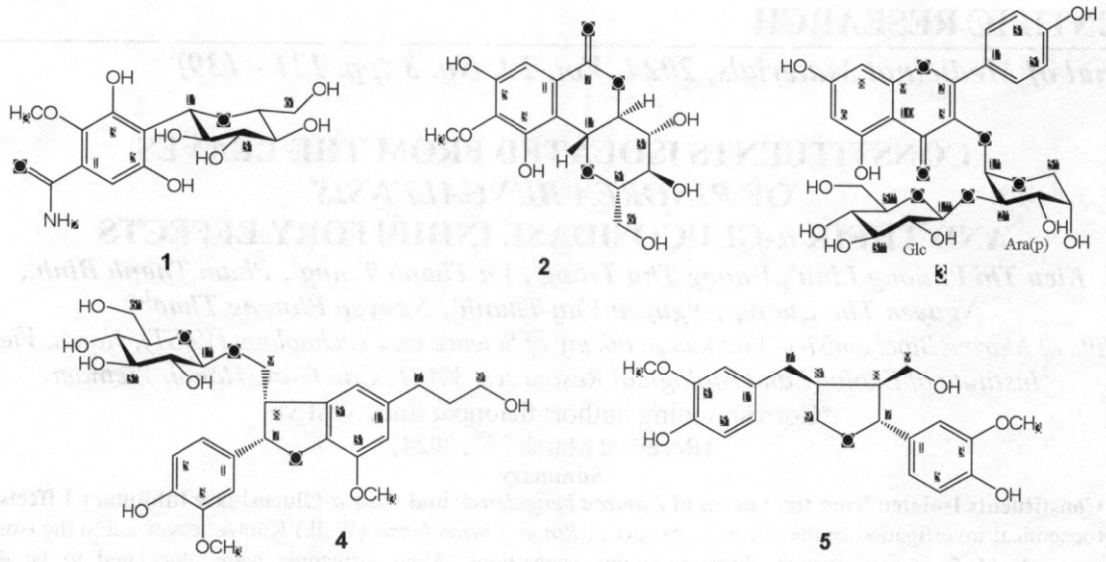

Fig. 1. Chemical structures of compounds **1**−**5**.

2.2. Plant material

The sample of the leaves of *R. bengalensis* was collected at Cam Ranh, Khanh Hoa province, Vietnam, in March 2023. The species was identified by Dr. Nguyen The Cuong. A voucher herbarium specimen has been deposited at the Herbarium of the Institute of Marine Biochemistry (VAST, Hanoi, Vietnam) with the accession number KH24.

2.3. Extraction and isolation

The leaves of *R. bengalensis* were removed from the stem, and foreign materials were separated. They were then dried in shade at room temperature until constant weight (1.5 kg) and powdered and extracted exhaustively by maceration in 80% EtOH (3×10 L, each 48h). The resulting solution was then filtered, and the filtrate was evaporated to dryness under reduced pressure at 50°C to afford 115.5 g of an organic extract. The residue was suspended in water and partitioned with *n*-hexane, CH_2Cl_2, and EtOAc (1×1 L of each, three times), obtaining *n*-hexane (43.6 g), CH_2Cl_2 (9.5 g), and EtOAc (11.6 g) fractions and a water layer.

The CH_2Cl_2 fraction was subjected to *silica gel* MPLC (column: Biotage SNAP Cartridge, KP-SIL, 50 g) using a mobile phase of *n*-hexane-acetone (0-5 min 10% acetone, 6-40 min 10-20% acetone, 41-85 min 50% acetone, 85-95 min 100% acetone, 15 mL/min, 95 min) to obtain ten fractions (D1-D10). Fraction D7 (0.3 g) was chromatographed over a *silica gel* column and eluted with a gradient of CH_2Cl_2-EtOAc (3:1, v/v) to yield three pooled subfractions (D7.1-D7.3). Of these, subfraction D7.1 (0.08 g) was further chromatographed over a *silica gel* column, eluted with *n*-hexane-CH_2Cl_2-EtOAc (1:1:0.05, v/v), and purified by separation over an RP-18 column, eluted with MeOH-H_2O (1.5:1, v/v) to afford compound **5** (2.0 mg).

The EtOAc fraction was initially separated by *silica gel* CC, eluted with different gradients of CH_2Cl_2-MeOH (from 100:0 to 0:100, 1.0 L of each) as mobile phase to give eight fractions (E1-E8) based on their TLC profiles. Fraction E4 (0.75 g) was subjected to a Sephadex LH-20 column using MeOH-H_2O (1:2, v/v) as the eluent to give five subfractions (E4.1-E4.5). Among them, subfraction E4.3 (0.08 g) was repeatedly purified using *silica gel* with EtOAc-MeOH (15:1, v/v) as the eluent and then RP-18 CC (MeOH-H_2O, 1:1, v/v) to give compounds **1** (1.3 mg) and **4** (2.5 mg). When the same steps were repeated as above, fraction E7 (0.58 g) was subjected to passage over a Sephadex LH-20 column (MeOH-H_2O, 1:2, v/v) to yield compound **3** (3.3 mg).

The water layer was subjected to separation over a Diaion HP-20 column with gradient elution using MeOH-H_2O mixtures (0:100 → 30:70 → 60:40, v/v) to obtain three major fractions (W1-W3). Fraction W2 (6.2 g) was successively separated on *silica gel* MPLC (column: Biotage SNAP Cartridge, KP-SIL, 50 g) using a mobile phase of CH_2Cl_2-MeOH (0-5 min 25% MeOH, 6-50 min 25-50% MeOH, 56-80 min 90% MeOH, 81-95 min 100% MeOH, 15 mL/min, 95 min) to obtain six subfractions (W2.1-W2.6). Finally, subfraction W2.1 (0.18 g) was subjected to an RP-18 column, and eluted

with the mobile phase MeOH-H_2O (1:4, v/v), to obtain compound **2** (5.5 mg).

4-C-β-D-glucopyranosyl-3,5-dihydroxy-2-methoxybenzamide (1): Pale yellow, amorphous solid; $[\alpha]_D^{24}$ +58.1 (*c* 0.15, MeOH); IR (KBr) ν_{max} 3500, 3200, 1660, 1640, 1604, 1519, 1180, 1080, 1035 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ_H: 7.11 (1H, s, H-6), 4.97 (1H, d, *J* = 10.2 Hz, H-1'), 3.83 (1H, t, *J* = 9.0 Hz, H-2'), 4.09 (1H, t, *J* = 9.0 Hz, H-3'), 3.45 (1H, t, *J* = 9.0 Hz, H-4'), 3.68 (1H, m, H-5'), 4.06 (1H, br d, *J* = 12.0 Hz, H-6'a), 3.70 (1H, dd, *J* = 5.5, 12.0 Hz, H-6'b), and 3.92 (3H, s, 2-OC<u>H</u>$_3$); ^{13}C NMR (CD_3OD, 150 MHz) δ_C: 119.4 (C-1), 142.3 (C-2), 149.4 (C-3), 117.3 (C-4), 152.3 (C-5), 111.0 (C-6), 74.3 (C-1'), 75.6 (C-2'), 81.4 (C-3'), 71.9 (C-4'), 83.0 (C-5'), 62.6 (C-6'), 165.7 (1-<u>C</u>ONH$_2$), and 60.8 (2-O<u>C</u>H$_3$); ESI-MS *m/z* 346 $[M + H]^+$.

Bergenin (2): White, amorphous powder; mp.: 238-240°C; $[\alpha]_D^{24}$ -55.8 (*c* 0.15, MeOH); IR (KBr) ν_{max}: 3423, 3389, 3247, 3204, 2951, 2896, 1701, 1613, 1464, 1348, 1335, 1234, 1092, 1071, 991, 858, 765 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ_H: 7.10 (1H, s, H-6), 4.97 (1H, d, *J* = 10.2 Hz, H-1'), 4.08 (1H, dd, *J* = 9.5, 10.2 Hz, H-2'), 3.83 (1H, dd, *J* = 9.0, 9.5 Hz, H-3'), 3.45 (1H, dd, *J* = 9.0, 9.5 Hz, H-4'), 3.66 (1H, m, H-5'), 3.72 (1H, dd, *J* = 5.4, 12.0 Hz, H-6'a), 4.03 (1H, dd, *J* = 2.4, 12.0 Hz, H-6'b), and 3.92 (3H, s, 4-OC<u>H</u>$_3$); ^{13}C NMR (CD_3OD, 150 MHz) δ_C: 119.4 (C-1), 117.3 (C-2), 149.4 (C-3), 142.3 (C-4), 152.3 (C-5), 111.0 (C-6), 165.8 (C-7), 74.3 (C-1'), 75.6 (C-2'), 81.4 (C-3'), 71.9 (C-4'), 83.0 (C-5'), 62.7 (C-6'), and 60.9 (4-O<u>C</u>H$_3$).

Kaempferol 3-O-β-D-glucopyranosyl-(1'''→2'')-O-α-L-arabinopyranoside (3): Pale yellow, amorphous solid; UV (MeOH) λ_{max}: 265, 346 nm; ^1H (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) spectroscopic data, see Table 1; ESI-MS *m/z* 603 $[M + Na]^+$.

(7α,8α)-Dihydrodehyrodiconiferyl alcohol-9-O-β-D-glucopyranoside (4): White, amorphous powder; $[\alpha]_D^{24}$ -73.2 (*c* 0.15, MeOH); UV (MeOH) λ_{max}: 279, 227, 203 nm; ^1H (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) spectroscopic data, see Table 2.

(+)-Lariciresinol (5): White, amorphous powder; mp.: 167-168°C; $[\alpha]_D^{24}$ +42.6 (*c* 0.15, MeOH); IR (KBr) ν_{max}: 3372, 2937, 2885, 1606, 1515, 1449, 1154, 1113, 1033 cm^{-1}; UV (MeOH) λ_{max}: 221, 282 nm; ^1H NMR (CD_3OD, 600 MHz) δ_H: 6.92 (1H, d, *J* = 1.8 Hz, H-2), 6.82 (1H, d, *J* = 7.8 Hz, H-5), 6.79 (1H, dd, *J* = 1.8, 7.8 Hz, H-6), 4.76 (1H, d, *J* = 7.2 Hz, H-7), 2.51 (1H, dd, *J* = 11.4, 13.2 Hz, H-8), 4.00 (2H, dd, *J* = 6.6, 8.4 Hz, H-9), 6.82 (1H, d, *J* = 1.8 Hz, H-2'), 6.74 (1H, d, *J* = 7.8 Hz, H-5'), 6.67 (1H, dd, *J* = 1.8, 7.8 Hz, H-6'), 2.96 (1H, dd, *J* = 4.8, 13.2 Hz, H-7'a), 2.39 (1H, m, H-7'b), 2.75 (1H, m, H-8'), 3.74 (1H, dd, *J* = 6.0, 8.4 Hz, H-9'a), 3.65 (1H, dd, *J* = 6.0, 10.8 Hz, H-9'b), 3.86 (3H, s, 3-OC<u>H</u>$_3$), and 3.85 (3H, s, 3'-OC<u>H</u>$_3$); ^{13}C NMR (CD_3OD, 150 MHz) δ_C: 133.6 (C-1), 113.5 (C-2), 149.0 (C-3), 145.8 (C-4), 116.2 (C-5), 122.2 (C-6), 84.1 (C-7), 43.9 (C-8), 73.5 (C-9), 135.8 (C-1'), 110.7 (C-2'), 149.0 (C-3'), 147.1 (C-4'), 116.0 (C-5'), 119.8 (C-6'), 33.7 (C-7'), 54.1 (C-8'), 60.5 (C-9'), 56.4 (3-O<u>C</u>H$_3$), and 56.4 (3'-O<u>C</u>H$_3$); ESI-MS *m/z* 383 $[M + Na]^+$.

2.4. α-Glucosidase inhibitory assay

As described in our early work, the bioassay was conducted using a 96-well plate, and the absorbance was determined at 405 nm using an Epoch microplate reader (Agilent BioTek, US). The control was prepared by adding phosphate buffer instead of the sample in the same way as the test. The blank was prepared by adding phosphate buffer instead of the α-glucosidase. Acarbose (95%, Thermo Scientific Chemicals, 459080010) was used as a positive control for the assay. The percentage of α-glucosidase enzyme inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right] \times 100$$

where % inhibition is the percentage of inhibition, A_{sample} is the corrected absorbance of the extracts or isolated compounds under testing $[A_{\text{sample(initial)}} - A_{\text{blank-sample}} - A_{\text{blank-plate}}]$, and A_{control} is the absorbance of the negative control $[A_{\text{negative-control-(initial)}} - A_{\text{blank-plate}}]$.

The nonlinear regression analysis was performed using GraphPad Prism version 6.0 for Windows. All compounds tested were reasonably pure according to their NMR spectra.

2.5. Kinetic study of α-glucosidase inhibition

The α-glucosidase inhibition mode of compound **1** was determined by Lineweaver-Burk plots. α-Glucosidase enzyme inhibition kinetic study was performed using a modified method previously explained elsewhere [7]. The kinetic analyses were performed at the six different concentrations of *p*-NPG (0.25, 0.5, 1.0, 2.0, 2.5, and 5.0 mM), four various concentrations of **1** (0.0, 10, 25, and 50 μM), and the fixed enzyme concentration (0.1 unit/L), and the three concentrations of each effective sample were evaluated for the manner of inhibition. The

second plot of the apparent K_m/V_{max} or $1/V_{max}$ versus the concentration of the inhibitor determined the inhibition constant. The type of inhibition was determined by regression analysis, using the following equation:

$$V = \frac{V_{max}S}{K_m\left(1 + \frac{[I]}{K_i}\right) + S\left(1 + \frac{[I]}{\alpha K_i}\right)}$$

where V represents the initial velocity in the presence or absence of the inhibitor, K_m is the Michaelis-Menten constant, V_{max} represents the maximum velocity, S and I are the concentrations of the substrate and inhibitor, K_i is the competitive inhibition constant and αK_i is the noncompetitive inhibition constant. The nonlinear regression analysis was performed using GraphPad Prism version 6.0 for Windows.

3. Results and discussion

Compound **1** was isolated as a pale yellow, amorphous powder, with a positive optical rotation { $[\alpha]_D^{24}$ +58.1 (*c* 0.15, MeOH)}. Its molecular formula was found to be $C_{14}H_{19}NO_9$ as inferred from the ESI-MS ion peak at *m/z* 346 $[M + H]^+$ and the ^{13}C NMR data. The ^1H, ^{13}C NMR and HSQC spectrum of **1** revealed 14 carbon signals, six of which were assigned to aromatic carbons (δ_C 111.0 to 152.3), one amide group [δ_C 165.7 (1-$\underline{C}ONH_2$)], and one methoxy group [δ_C 60.8/δ_H 3.92 (3H, s, 2-$OC\underline{H}_3$)]. The presence of one singlet signal [δ_H 7.11 (1H, H-6)/δ_C 111.0 (C-6)] and five non-protonated aromatic carbons [δ_C 119.4 (C-1), 142.3 (C-2), 149.4 (C-3), 117.3 (C-4), and 152.3 (C-5)], were in agreement with a pentasubstituted aromatic ring in **1**. Additionally, a set of complex signals of the oxygenated carbons was also present [δ_C 74.3 (C-1'), 75.6 (C-2'), 81.4 (C-3'), 71.9 (C-4'), 83.0 (C-5'), and 62.6 (C-6')]. Moreover, analysis of the NMR spectroscopic data of **1** revealed the presence of a *C*-glucosidic moiety characterized by an anomeric proton [δ_H 4.97 (1H, d, J = 10.2 Hz, H-1')], as well as an anomeric carbon signal [δ_C 74.3 (C-1')] in the HSQC spectrum. This was supported by the presence of three triplets and one multiplet of one proton each attributed to the four oxymethines in the glucosidic moiety [δ_H 3.83 (1H, t, J = 9.0 Hz, H-2'), 4.09 (1H, t, J = 9.0 Hz, H-3'), 3.45 (1H, t, J = 9.0 Hz, H-4'), and 3.68 (1H, m, H-5'], and the signals of one oxymethylene [δ_H 4.06 (1H, br d, J = 12.0 Hz, H-6'a) and 3.70 (1H, dd, J = 5.5, 12.0 Hz, H-6'b)]. The large coupling constant of the anomeric proton (^3J = 10.2 Hz) of H-1'/Glc with H-2'/Glc established the β-linkage of the D-glucopyranose moiety. Confirmation of the partial structural units and determination of their connectivity was established by the cross-peaks in the HMBC spectrum that were observed between the anomeric proton δ_H 4.97 (H-1') with δ_C 149.4 (C-3), 117.3 (C-4), and 152.3 (C-5) confirming the glycosidic linkage of the glucose unit at C-4. This information is clearly expressed in the downfield shift of an aromatic non-protonated carbon C-4 (δ_C 117.3) in the pentasubstituted aromatic ring. Furthermore, the HMBC correlations between the aromatic proton at δ_H 7.11 (H-6) with δ_C 164.7 (1-$\underline{C}ONH_2$), C-2, C-4, C-5, and between δ_H 3.92 (2-$OC\underline{H}_3$) with C-2 confirmed the methoxy group at C-2 of the benzamide moiety (Fig. 2). Based on the above evidence and comparison with reported literature [8], compound **1** was finally concluded to be 4-*C*-β-D-glucopyranosyl-3,5-dihydroxy-2-methoxybenzamide. This compound was previously obtained from the stem barks of *Piper guineense* (family Piperaceae) [8].

Compound **2** was obtained as a white, amorphous powder. The ^1H NMR spectrum of **2** confirmed the presence of one characteristic singlet in a pentasubstituted aromatic ring [δ_H 7.10 (1H, s, H-6) beside the peaks [δ_H 4.97 (1H, d, J = 10.2 Hz, H-1'), and 3.45-4.08] characteristic of hydrogens attached to oxygenated carbons, indicating the presence of a glucoside moiety. The glucoside moiety was assigned by coupling constants observed by the ^1H NMR data and the chemical shifts in the ^{13}C NMR spectrum. The comparison of the NMR data with NMR spectroscopic data in the published literature [9],[10] supported the identification of **2**. Thus, compound **2** was established bergenin. According to the Merck Index, bergenin (**2**) was originally isolated from the rhizomes *Bergenia* (syn. *Saxifranga*) *crassifolia* (Saxifragaceae) by Garreau et al. [11]. To date, bergenin has been obtained from different species of different families (at least 112 species distributed in 34 plant families) [12],[13]. Compound **3** was obtained as a pale yellow, amorphous solid. Its molecular formula $C_{26}H_{28}O_{15}$ was determined by the ESI-MS ion peak at *m/z* 603 $[M + Na]^+$ and the ^{13}C NMR data. The ^1H NMR spectroscopic data of **3** exhibited the characteristic peaks for a flavone skeleton. The signals at δ_H 6.22 (1H, br s) and 6.41 (1H, br s) were indicative of the A-ring protons, while the signals at δ_H 8.03 (2H, d, J = 8.4 Hz), and 6.94 (2H, d, J = 8.4 Hz) revealed an A_2B_2 spin system, corresponding to 1',4'-substituted flavonoid B-ring. Additionally, two

anomeric proton signals of a disaccharide moiety were also observed at δ_H 5.50 (1H, d, J = 3.6 Hz, H-1") and 4.58 (1H, d, J = 7.8 Hz, H-1'") that displayed HSQC correlations with anomeric carbon signals at δ_C 101.2 (C-1") and 105.3 (C-1'") (Table 1). The β-anomeric configuration for the D-glucopyranosyl unit was determined by their $^3J_{H\text{-}1'''/H\text{-}2'''}$ coupling constant of ~7-8 Hz, and the α-anomeric configuration for the L-arabinopyranosyl unit was determined according to the $^3J_{H\text{-}1''/H\text{-}2''}$ coupling constant of 3.6 Hz. The ^{13}C NMR and HSQC data showed 26 carbon signals, 12 of which were assigned to the aromatic carbons and three to *C*-ring resonances [δ_C 179.6 (C-4), 158.5 (C-2), and 135.6 (C-3)] of kaempferol aglycone. The glycosylation position of **3** was reasonably assigned to C-3 rather than other positions of the kaempferol aglycone based on the downfield shift of one tertiary oxygenated carbon (δ_C 135.6). HMBC correlations confirmed the link between the anomeric proton (δ_H 5.50) of the arabinosyl moiety and C-3 (δ_C 135.6) of kaempferol. There was an HMBC correlation between the anomeric proton of the glucosyl moiety (δ_H 4.58) and C-2" (δ_C 80.0), revealing glycosylation at this position. Additionally, both C-2/H-2 of the arabinose unit were deshielded (δ_C 80.0/δ_H 4.23), and the carbon correlated with the glucosyl anomeric proton at δ_H 4.58, confirming the C-1'"→C-2" inter-glycosidic linkage between the two sugar moieties (Fig. 2). Based on the above evidence and comparison with reported literature [14], compound **3** was finally concluded to be kaempferol 3-*O*-β-D-glucopyranosyl-(1'"→2")-*O*-α-L-arabinopyranoside. This compound was previously obtained from the leaves of *Brugmansia suaveolens* (family Solanaceae) [14]. However, no studies regarding the biological activities of this compound have been reported to date.

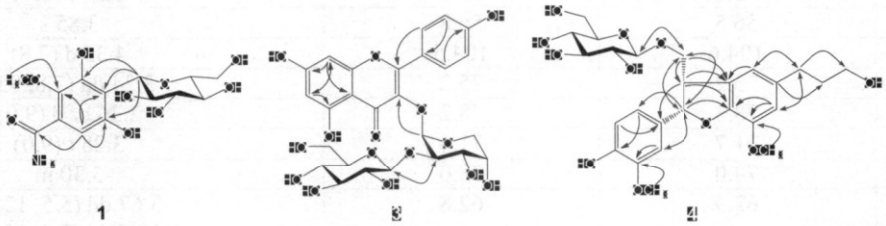

Fig. 2: Key HMBC correlations of **1**, **3**, and **4**.

Table 1. ^{1}H and ^{13}C NMR spectroscopic data for **3** and the reference compound.

Pos.	$^{\#}\delta_C$ [a]	**3**	
		δ_C [a,b]	δ_H [a,c] mult. (J in Hz)
2	158.5	158.5	-
3	135.7	135.6	-
4	179.7	179.6	-
5	161.6	161.6	-
6	99.9	100.0	6.22 br s
7	166.0	166.2	-
8	94.7	94.8	6.41 br s
9	159.0	158.9	-
10	105.8	105.7	-
1'	122.6	122.6	-
2', 6'	132.4	132.3	8.03 d (8.4)
3', 5'	116.5	116.4	6.94 d (8.4)
4'	163.1	163.1	-
1"	101.3	101.2	5.50 d (3.6)
2"	80.1	80.0	4.23 dd (3.6, 5.4)
3"	71.3	71.3	3.98 dd (5.4, 6.4)
4"	66.6	66.6	3.88 m
5"	63.4	63.4	3.72 m/3.25 m
1'"	105.4	105.3	4.58 d (7.8)
2'"	75.2	75.2	3.24 dd (7.8, 9.0)
3'"	78.1	78.3	3.40 dd (9.0, 9.0)
4'"	71.4	71.3	3.33 t (9.0)
5'"	78.0	78.0	3.35 m
6'"	62.7	62.7	3.81 dd (1.8, 12.0) 3.73 dd (4.5, 12.0)

Measured in [a]CD_3OD, [b]125 MHz, and [c]600 MHz. $^{\#}\delta_C$ of kaempferol 3-*O*-β-D-glucopyranosyl-(1'"→2")-*O*-α-L-arabinopyranoside [14].

Table 2. ^{1}H and ^{13}C NMR spectroscopic data for **4** and the reference compound.

Pos.	$^{\#}\delta_C$ [a]	4	
		δ_C [a,b]	δ_H [a,c] mult. (*J* in Hz)
1	134.8	134.7	-
2	110.9	110.8	6.99 d (1.8)
3	149.0	149.0	-
4	147.5	147.4	-
5	116.1	116.1	6.77 d (8.0)
6	119.8	119.7	6.87 dd (1.8, 8.0)
7	89.0	88.9	5.58 d (6.0)
8	53.3	53.2	3.64 m
9	72.4	72.4	3.75 m 4.20 dd (5.4, 9.6)
3-OCH_3	56.4	56.4	3.82 s
1'	137.0	136.9	-
2'	114.4	114.2	6.72 d (1.8)
3'	145.3	145.2	-
4'	147.5	147.5	-
5'	129.7	129.7	-
6'	118.3	118.2	6.83 d (1.8)
7'	32.9	32.9	2.62 t (7.2)
8'	35.8	35.8	1.81 m
9'	62.3	62.2	3.56 t (6.6)
3'-OCH_3	56.5	56.8	3.85 s
1"	104.6	104.5	4.35 d (7.8)
2"	75.2	75.2	3.24 dd (7.8, 9.0)
3"	78.3	78.2	3.36 br d (9.0)
4"	71.7	71.6	3.28 t (9.0)
5"	78.0	78.0	3.30 m
6"	62.9	62.8	3.67 dd (5.5, 12.0) 3.87 dd (2.5, 12.0)

Measured in [a]CD_3OD, [b]125 MHz, and [c]600 MHz. $^{\#}\delta_C$ of (7*α*,8*α*)-dihydrodehyrodiconiferyl alcohol-9-*O*-*β*-D-glucopyranoside [16].

Compound **4** was isolated as a white, amorphous powder. The ^{1}H NMR spectrum of **4** exhibited the presence of one ABX spin system of one 1,3,4-trisubstituted aromatic ring [δ_H 6.99 (1H, d, J = 1.8 Hz), 6.77 (1H, d, J = 8.0 Hz), and 6.87 (1H, dd, J = 1.8, 8.0 Hz)], and one A_2 spin system of one 1',3',4',5'-tetrasubstituted aromatic ring [δ_H 6.72 (1H, d, J = 1.8 Hz) and 6.83 (1H, d, J = 1.8 Hz)], as well as the signal corresponding to an anomeric proton [δ_H 4.35 (1H, d, J = 7.8 Hz)], and two oxymethylene protons [δ_H 3.87 (1H, dd, J = 12.0, 2.5 Hz) and 3.67 (1H, dd, J = 12.0, 5.5 Hz)] that were attributed to H-1' and H_2-6" of a glucopyranosyl moiety, respectively. Besides, one oxygenated methine [δ_H 5.58 (1H, d, J = 6.0 Hz, H-7)], two oxygenated methylenes [δ_H 4.20 (1H, dd, J = 5.4, 9.6 Hz, H-9a)/3.75 (1H, m, H-9b) and 3.56 (2H, t, J = 6.6 Hz, H-9')], one methine [δ_H 3.64 (1H, m, H-8)], two methylenes [δ_H 2.62 (1H, t, J = 7.2 Hz, H-7'), 1.81 (1H, m, H-8')], and two methoxy groups [δ_H 3.82 (3H, s, 3-$OC\underline{H}_3$) and 3.85 (3H, s, 3'-$OC\underline{H}_3$)], were also observed (Table 2). Their corresponding six carbon signals [δ_C 88.9 (C-7), 53.2 (C-8), 72.4 (C-9), 32.9 (C-7'), 35.8 (C-8'), and 62.2 (C-9')] were identified based on the HSQC spectrum. The ^{13}C NMR spectrum of **4** showed 26 carbon signals, of which 12 aromatic carbons in the downfield, two methoxy groups, and the remaining six carbons were assigned to a glucosyl moiety (Table 2). The above spectroscopic data revealed that compound **4** should be a dihydrobenzofuran neolignan glycoside. A dihydrobenzofuran neolignan fragment was constructed from the HMBC correlations of H-7 with C-2, C-6, C-9, C-4'; H-8 with C-1, C-4', C-5'; and H-7' with C-2', C-6', C-9'. Oxygenated methylene protons at δ_H 3.75/4.20 (H-9) showed HMBC correlations with C-7, C-8 and C-5' of the dihydrobenzofuran and C-1" of the *β*-D-glucopyranoside and cross-peak from δ_H 4.35 (H-1") with δ_C 72.4 (C-9) (Fig. 2), suggesting that the *β*-D-glucopyranosyl part is

connected to the dihydrobenzofuran nucleus through the oxymethylene group. Additionally, the assignment of the methoxy groups was completed by the observed key HMBC interactions from protons δ_H 3.82 to δ_C 149.0 (C-3) and δ_H 3.85 to δ_C 145.2 (C-3'). The C-7/C-8 relative configuration was assigned as *trans* from the small coupling constant (6.0 Hz, **4**; 7.4 Hz, *trans*-form; 8.4 Hz, *cis*-form) [15]. Furthermore, relative configurations at C-7 and C-8 were deduced to be 7α and 8α, respectively, by comparing their carbon chemical shifts (δ_{C-7} 88.9 and δ_{C-8} 53.2) with those reported in the literature (relative 7α,8α isomer [16]: δ_{C-7} 89.0 and δ_{C-8} 53.3), relative 7β,8α isomer [17]: δ_{C-7} 83.8 and δ_{C-8} 54.1). A neolignan with a molecular structure identical to that of **4** has previously been reported and named (7α,8α)-dihydrodehyrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside [16].

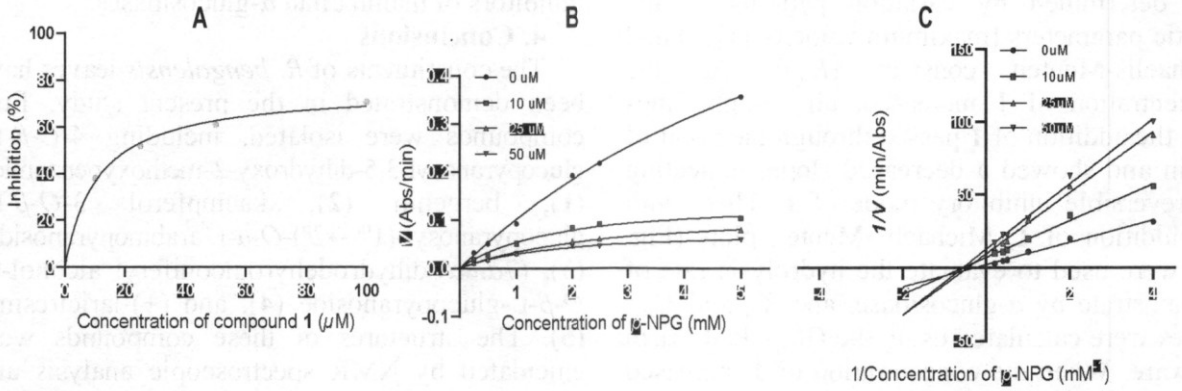

Fig. 3: Inhibitory effect of **1** on α-glucosidase enzyme (A); Michaelis-Menten plot of **1** (B); and Lineweaver-Burk plot for α-glucosidase inhibition by **1** (C).

Compound **5** was also obtained as a white, amorphous powder. Its molecular formula was established as $C_{20}H_{24}O_6$ by the ESI-MS ion peak at *m/z* 383 $[M + Na]^+$. Its ^{1}H NMR spectrum were attributed to two 1,3,4-trisubstituted aromatic rings (δ_H 6.67-6.92), one benzylic oxymethine [δ_H 4.76 (1H, d, J = 6.0 Hz, H-7)], two oxymethylenes [δ_H 4.00 (2H, dd, J = 6.6, 8.4 Hz, H-9), 3.74 (1H, dd, J = 6.0, 8.4 Hz, H-9'a)/3.65 (1H, dd, J = 6.0, 10.8 Hz, H-9'b)], two methines [δ_H 2.51 (1H, dd, J = 11.4, 13.2 Hz, H-8) and 2.75 (1H, m, H-8'), one methylene [δ_H 2.96 (1H, dd, J = 4.8, 13.2 Hz, H- 7'a) and 2.39 (1H, m, H-7'b)], and two methoxy groups [δ_H 3.85 (3H, s, 3-OC<u>H</u>$_3$) và 3.86 (3H, s, 3'-OC<u>H</u>$_3$)]. The ^{13}C NMR spectrum showed signals for 12 aromatic carbons (δ_C 110.7-149.0), one oxymethine [δ_C 33.7 (C-7)], two oxymethylenes [δ_C 73.5 (C-9) and 60.5 (C-9')], two methines [δ_C 43.9 (C-8) and 54.1 (C-8')], one methylene [δ_C 84.1 (C-7')], and two methoxy groups [δ_C 56.4 (3-O<u>C</u>H$_3$) and 56.4 (3'-O<u>C</u>H$_3$)]. These were characteristic of those reported for tetrahydrofuranoid-type lignans. A detailed investigation of NMR spectroscopic data supported the identification of **5** and in comparison with NMR spectroscopic data in the published literature [18], compound **5** was established (+)-lariciresinol. Previously, (+)-lariciresinol was reported from the fresh stem of *Aquilaria sinensis* and found to display moderate to weak growth inhibitory effects against SGC-7901 and SMMC-7721 cell lines [18].

All isolated compounds were initially evaluated for their inhibitory activity against α-glycosidase at 50 μM using acarbose as a positive control. Compounds with an inhibition rate higher than 50% (**1**, **4**, and **5**) were screened for half-maximal inhibitory concentration (IC_{50}). Among them, compounds **1** and **5** exhibited not only good α-glucosidase inhibitory activities with IC_{50} values of 11.57 ± 0.76 μM (Fig. 3A) and 14.22 ± 0.97 μM, respectively, but also better activity than the positive control, acarbose (with an IC_{50} value of 46.78 ± 1.37 μM). Meanwhile, compound **4** exhibited a moderate inhibitory effect against α-glucosidase with an IC_{50} value of 69.98 ± 1.36 μM. The remaining compounds **2** and **3** did not show α-glucosidase inhibitory activity when tested at concentrations below 200 μM.

To provide insights into the mechanism underlying this inhibition, a kinetic analysis was performed to visualize the nature of the binding between the α-glucosidase and compound **1** using Lineweaver-Burk plots. Measurement of the

effect of varying substrate concentration with an inhibitor at several concentrations produces a group of double reciprocal curves, each with a characteristic slope and *y*-intercept. The changes in slope and *y*-intercept are characteristic of the different types of inhibitors. Because compound **1** was the most potent inhibitor against α-glucosidase, its inhibitory mechanism was investigated. Firstly, the plots of *v* versus the concentrations of the concentrations α-glucosidase were conducted. The inhibition mode was determined by variation patterns of the kinetic parameters [maximum velocity (V_{max}) and Michaelis-Menten constant (K_m)]. As the concentration of **1** increased, all straight lines with the addition of **1** passed through the point of origin and showed a decreased slope, indicating the reversible inhibitory mode of **1**. Then, with the addition of **1**, Michaelis-Menten plots (Fig. 3B) were used to evaluate the hydrolysis rate of the substrate by α-glucosidase, and K_m and V_{max} values were calculated using the GraphPad Prism software. When the concentration of **1** increased from 10 to 50 μM, the K_m values increased from 1.67 ± 0.56 to 2.70 ± 0.56 μM, whereas the V_{max} value decreased from 0.14 ± 0.04 to 0.08 ± 0.01 μM, suggesting the mixed inhibition mode of **1** on α-glucosidase. Therefore, kinetic analysis indicates that compound **1** interaction can occur in the free enzyme and also in the enzyme-substrate complex at a site that is distinct from the catalytic site, thus inducing changes in the shape of the active site such that the substrate will no longer fit well. The Lineweaver-Burk plots (Fig. 3C) present the changes in the slope and *y*-intercept characterized at each concentration of **1** that also suggested the mixed inhibition type on α-glucosidase (K_i = 21.37 ± 0.08 μM). The mixed-type inhibition with obvious selectivity hinted at an allosteric effect. The validation of the antidiabetic activity will require further investigation of mammalian enzymes, although phenolics are well-known inhibitors of mammalian α-glucosidases.

4. Conclusions

The constituents of *R. bengalensis* leaves have been demonstrated in the present study. Five compounds were isolated, including 4-*C*-β-D-glucopyranosyl-3,5-dihydroxy-2-methoxybenzamide (**1**), bergenin (**2**), kaempferol 3-*O*-β-D-glucopyranosyl-(1'''→2'')-*O*-α-L-arabinopyranoside (**3**), (7α,8α)-dihydrodehyrodiconiferyl alcohol-9-*O*-β-D-glucopyranoside (**4**), and (+)-lariciresinol (**5**). The structures of these compounds were elucidated by NMR spectroscopic analysis and comparison with the reported data. Furthermore, the α-glucosidase properties of all isolated compounds were demonstrated *in vitro*. This is the first time that chemical constituents and their α-glucosidase inhibitory activity were reported from *R. bengalensis*.

Acknowledgments: *This research is funded by the Vietnam Academy of Science and Technology (Under grant number: UQĐTCB.04/22-24).*

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Journal of Medicinal Materials, 2024, Vol. 29, No. 3 (pp. 139 - 143)

CHEMICAL CONSTITUENTS OF THE LEAVES OF *PLANTAGO MAJOR* L.

Nguyen Thi Duyen[1,] *, Hoang Thi Ngoc Anh[2], Nguyen Minh Toan[1], Tran Trung Hieu[3], Bui Thi Thai Linh[4], Nguyen Van Tai[1]

[1]National Institute of Medicinal Materials, Hanoi, Vietnam; [2]Dai Nam University, Hanoi, Vietnam; [3]Vinh University, Vietnam; [4]Hanoi University of Science, Vietnam

*Corresponding author: nguyenduyen6784@gmail.com

(Received May 14th, 2024)

Summary

Chemical Constituents of the Leaves of *Plantago major* L.

From ethyl acetate and *n*-butanol extracts of the leaves of *Plantago major* L., seven compounds (**1**-**7**) were isolated. Their chemical structures were identified as 5-(hydroxymethyl)furan-2-carbaldehyde (**1**), vanillic acid (**2**), uracil (**3**), daucosterol (**4**), baicalein (**5**), scutellarein-7-*O*-*β*-D-glucopyranoside (**6**) and oct-l-en-3-*O*-*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside (**7**), based on spectral data and literature references. Compounds **1**, **3**, **6**, and **7** were isolated from this plant for the first time.

Keywords: *Plantago major, Scutellarein-7-O-β-D-glucopyranoside, Oct-1-en-3-O-β-D-xylopyranosyl-(1→6)-β-D-glucopyranoside.*

1. Introduction

Plantago major L. belongs to the Plantaginaceae family and is widely cultivated in Vietnam for its medicinal properties, including diuretic effects, and treatment for long coughs, tracheitis, dysentery, and red eye [1]. Scientific studies *in vivo* and *in vitro* have shown wound healing effects, antidiabetic, antidiarrhoeal, anti-inflammatory, antinociceptive, antioxidant and free radical scavenger, anticancer, antibacterial, and antiviral [2],[3],[4],[5],[6],[7],[8]. The up-to-date phytochemical studies on the plant have resulted in the identification of flavonoids, alkaloids, terpenoids, phenolics, iridoid glycosides, fatty acids, polysaccharides, and vitamins [8],[9],[10],[11],[12],[13],[14],[15]. In this study, we isolated and identified seven compounds from the leaves of *Plantago major*: 5-(hydroxymethyl)furan-2-carbaldehyde (**1**), vanillic acid (**2**), uracil (**3**), daucosterol (**4**), baicalein (**5**), scutellarein-7-*O*-*β*-D-glucopyranoside (**6**), and oct-l-en-3-*O*-*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside (**7**) (Fig.1). Among them, compounds **1**, **3**, **6** and **7** were isolated from this plant for the first time.

Materials and methods

2.1. Plant material

The samples were collected at the Hanoi Research Centre for Cultivation and Processing of Medicinal Plants in May 2023. The plant was identified *Plantago major* L., Plantagineceae by MSc. Nguyen Van Hieu and MSc. Nguyen Quynh Nga. A voucher specimen (PM52023) was deposited at the National Institute of Medicinal Materials.

2.2. General experiment procedures

The NMR measurements were conducted using CD_3OD, $CDCl_3$, and DMSO-d_6 as solvents on a Bruker NMR spectrometer with a frequency of 600 MHz. Tetramethylsilane was used as an internal standard, and chemical shifts were reported in δ (ppm). Electrospray Ionization Mass Spectrometry (ESI-MS) was performed using an Agilent 1100 series LC-MSD ion trap spectrometer. Column chromatography was performed using *silica gel* (70-230 or 230-400