RESEARCH ON PHYTOCHEMICAL AND ALFA-GLUCOSIDASE INHIBITORY ACTIVITY OF ETHYL ACETATE FRACTION OF RUELLIA TUBEROSA

Pham Thi Nhat Trinh¹, Pham Ngoc An², Nguyen Trong Tuan³, Nguyen Thi Le Thuy⁴, Tong Thanh Danh⁵, Le Tien Dung²,6,*

¹Faculty of Natural Sciences, Tien Giang University, 119 Ap Bac – My Tho – Tien Giang
²Institute of Applied Materials Science, VAST, 01A TL29, Dist.12, Ho Chi Minh city
³Department of Chemistry, College of Natural Sciences, Can Tho University, Dist. Ninh Kieu, Can Tho
⁴Department of Food Technology, Faculty of Biotechnology, Open University Ho Chi Minh City, 97 Vo Van Tan, Dist. 3, Ho Chi Minh City
⁵Department of Organic Chemistry, Faculty of Chemical Engineering, Ho Chi Minh City University of Technology, 268 Ly Thuong Kiet, Dist. 10, Ho Chi Minh City
⁶Graduate University of Science and Technology, VAST, 18 Hoang Quoc Viet, Cau Giay, Ha Noi

*Email: inpcdung@yahoo.com

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ABSTRACT

Ruellia tuberosa L. is a folk remedy, which is used in the treatment of diabetes mellitus, antiinflammation and diuretic agent. However, its α-glucosidase inhibitory activity has not been investigated so far. In the present study, the antidiabetic activities of the hexane, ethyl acetate and methanol fraction of crude ethanolic extract of this plant were investigated. Our preliminary results showed that the crude ethanol extract as well as ethyl acetate and methanol fractions of Ruellia tuberosa showed α-glucosidase inhibitory activity with IC₅₀ of 15.84, 4.73 and 8.27 µg/ml, respectively. From the ethyl acetate extract of whole plant of Ruellia tuberosa, four compounds, including rutin (1), syringaresinol (2), catechin (3) and pulmatin (4) were isolated and identified by column chromatography. Their structures were elucidated by spectroscopic method including MS, 1D and 2D- NMR and comparison with the literature values.

Keywords: Ruellia tuberosa L., flavonoid, anthraquinone glycoside, α-glucosidase inhibitory activity.

1. INTRODUCTION

Diabetes mellitus is a chronic disease which relates to hyperglycemia as a result of defects in insulin secretion, insulin action, or both. The goal of treatment of diabetes is to control and keep blood glucose levels at normal or near-normal levels, to reduce postprandial blood glucose
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To control blood glucose, people are used to drugs, including inhibitors α-glucosidase enzyme. The α-glucosidase inhibitors prevent the digestion of carbohydrates, lead to reduce postprandial absorption of glucose, thereby delaying the increase in blood glucose. Public interest in alternative therapies, including the use of plants and natural dietary supplements, has risen throughout the world. Ruellia tuberosa L., is widely distributed in Vietnam, and is a folk remedy in the treatment of diabetes mellitus, antihypertensive, analgesic, anti-inflammatory and diuretic agent.

Ruellia tuberosa L. possessed significant blood glucose lowering effect in alloxan-induced diabetic rat and rabbit [3, 4], antioxidant, xanthine oxidase inhibitory [5], anticholinesterase [6], hypolipidemic [7], antiinociceptive and anti-inflammatory [8] activities. It was reported that five flavonoids including: cirsimaritin, cirsimaritin, cirsiliol 4-glucoside, sorbifolin, and pedalin along with betulin, vanillic acid, and indole-3-carboxaldehyde were isolated from the ethyl acetate fraction of methanolic extracts of R. tuberosa L. [9]. Phenylethanoid and flavone glycosides included apigenin, 3,5-diglucoside, apigenin-7-O-glucuronide, apigenin glucoside, apigenin rutinoside, luteolin glucoside, verbascoside, isoverbascoside, nuomioside were also reported in R. tuberosa L. [10-12]. In addition, our previously published result showed that the main chemical components were flavonoid and phenyl ethanoid [13]. The aim of this present work is to evaluate the inhibition of α-glucosidase and isolate phytochemicals from active fraction of Ruellia tuberosa.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Solvents: n-hexane, chloroform, ethyl acetate, methanol and ethanol 96 % were purchased from Chemsol company (Viet Nam). Silica gel 60 (0.063–0.200 mm, Merck) and silica gel C-18 were used for column chromatograph. TLC F254 plate (Merck) and TLC RP-18 F254 plate (Merck) were used for thin layer chromatography. NMR spectra were recorded on a Bruker AM500 FT-NMR Spectrometer of Institute of Chemistry, VAST.

2.2. Sample treatment and preparation

Ruellia tuberosa L. were collected at July, 2017 in My Tho, Tien Giang, Vietnam. The plants were authenticated and voucher specimens (S072017) has been preserved in the Institute of Applied Materials Science. The whole plants were washed away from muds and dust; after that samples were dried in an oven at about 50 ºC until well-dried.

2.3. Extraction and isolation

The dried powder of whole plant (5 kg) was soaked in 96% ethanol at room temperature for four times (4 × 20 L) and filtered. The filtrate was concentrated under reduced pressure to give crude ethanol extract (350 g). The extract was then fractionated over flash column chromatography eluting successively with hexane, ethyl acetate, and methanol, respectively to yield hexane (80 g), ethyl acetate (95 g), and methanol fractions (148 g).

The ethyl acetate fraction was subjected to a silica gel column chromatography and eluted with Hexane-EtOAc to obtain six subfractions (E1-6). Subfraction E1 was further separated on a silica gel CC and eluted under gradient hexane-EtOAc (from 30:1 to 10:1, v/v) to yield five
subfractions (E1.1-7). Fraction E1.2 was rechromatographed on silica gel CC, eluting with CHCl₃: MeOH (30:1 to 5:1, v/v) to obtain six subfractions (E1.2.1-6). Subfraction E1.2.4 was chromatographed on silica gel using CHCl₃: MeOH (8:1, v/v) as eluent, then purified by sephadex LH-20 (MeOH 100 %) to give compound 3 (13.8 mg). Subfraction E1.2.3 was further chromatographed on Rp18 CC, eluted with MeOH: H₂O (1:1, v/v) and was then subjected to a sephadex LH20 CC (CHCl₃:MeOH 1:1) to give compound 2 (5.6 mg).

Fraction E5 was further separated on a silica gel column, eluted with CHCl₃:MeOH (from 50:1 to 5:1, v/v) to yield nine subfractions (E5.1-9). Subfraction E5.4 was repeatedly chromatographed on silica gel CC Rp18 eluted with CHCl₃: MeOH (15:1 to 7:1, v/v) to obtain four subfractions (E5.4.1-4). The fraction E5.4.4 was further chromatographed on silica gel Rp18, eluted with MeOH:H₂O (1:3, v/v) to obtain three subfractions (E5.4.4.1-3). Fraction E5.4.1 was repeatedly separated on a silica gel Rp18 with MeOH: H₂O (1:4, v/v) to obtain compound 1 (17.2 mg). Fraction E4.4.3 was purified through a sephadex LH 20 column using MeOH as eluent to yield compound 4 (8.5 mg).

**Compound 1**  
1H-NMR (500 MHz, CD₃OD), δ (ppm), J (Hz): 6.22 (1H, s, H-6); 6.41 (1H, s, H-8); 7.68 (1H, s, H-2′); 6.90 (1H, d, 7.5Hz, H-5′); 7.65 (1H, d, 7.5Hz, H-6′); 5.13 (1H, d, 7.0Hz, H-1′′′): 4.54 (1H, s, H-1″″). 13C-NMR (125 MHz, CD₃OD), δ (ppm): 159.3 (C-2); 135.6 (C-3); 179.3 (C-4); 162.9 (C-5); 99.9 (C-6); 165.9 (C-7); 94.8 (C-8); 158.4 (C-9); 105.6 (C-10); 123.0 (C-1′); 117.6 (C-2′); 145.8 (C-3′); 149.7 (C-4′); 116.0 (C-5′); 123.5 (C-6′); 104.7 (C-1″); 75.7 (C-2″); 78.1 (C-3″); 71.3 (C-4″); 77.1 (C-5″); 68.5 (C-6″); 102.3 (C-1‴); 72.0 (C-2‴); 72.2 (C-3‴); 73.9 (C-4‴); 69.6 (C-5‴); 17.8 (C-6‴).

**Compound 2**  
ESI-MS: m/z 417.0[M-H]$.^1$H-NMR(500 MHz, acetone-d₆), δ (ppm), J (Hz): 6.68 (4H, s, H-2, H-2′, H-6, H-6′); 4.61 (2H, d, 4.0Hz, H-7, H-7′); 3.10 (2H, m, H-8, H-8′); 4.24 (2H, dd, 8.5 and 6.5 Hz, H-9a, H-9b); 3.87 (2H, dd, 3.0 and 9.0 Hz, H-9b, H-9a); 3.82 (12H, s, OCH$_3$10,10′,11, 11′). 13C-NMR (125 MHz, acetone-d₆), δ (ppm): 133.2 (C-1-C-1′); 104.4 (C-2, C-2′); 148.6 (C-3, C-3′); 136.2 (C-4, C-4′); 148.6 (C-5, C-5′); 104.4 (C-6, C-6′); 86.7 (C-7, C-7′); 55.3 (C-8, C-8′); 72.3 (C-9, C-9′); 56.6 (C-10, C-10′, C-11, C-11′).

**Compound 3**  
1H-NMR(500 MHz, DMSO-d$_6$), δ (ppm), J (Hz):4.59 (1H, d, 8.0 Hz, H-2); 4.01 (1H, m, H-3); 2.89 (1H, dd, 5.5, 16.0 Hz, H-4a); 2.55 (1H, dd, 8.5, 16.0 Hz, H-4e); 5.88 (1H, d, 2.5 Hz, H-6); 5.95 (1H, d, 2.5 Hz, H-8); 6.86 (1H, d, 2.0 Hz, H-2′); 6.79 (1H, d, 8.0, H-5′); 6.74 (1H, dd, 2.0, 8.0 Hz, H-6); 13C-NMR (125 MHz, DMSO-d$_6$), δ (ppm):32.8 (C-2); 68.8 (C-3); 28.5 (C-4); 157.5 (C-5); 95.5 (C-6); 156.9 (C-7); 96.3 (C-8); 157.8 (C-9); 100.8 (C-10); 132.2 (C-1′); 115.2 (C-2′); 146.2 (C-3′); 146.2 (C-4′); 116.1 (C-5′); 120.0 (C-6′).

**Compound 4**  
ESI-MS: m/z 416.91 [M+H]$^+$1H-NMR(500 MHz, DMSO-d$_6$), δ (ppm), J (Hz):7.71 (1H, s, H-2); 7.54 (1H, s, H-4); 7.66 (1H, dd, 8.0, 1.0 Hz, H-5); 7.74 (1H, t, 8.0 Hz, H-6); 7.35 (1H, dd, 8.0, 1.0 Hz, H-7); 2.47 (3H, s, H-3); 5.16 (1H, d, 7.5 Hz, H-1′). 13C-NMR (125 MHz, DMSO-d$_6$), δ (ppm): 158.4 (C-1); 122.8 (C-2); 147.4 (C-3); 121.3 (C-4); 118.3 (C-5); 136.2 (C-6); 124.3 (C-7); 187.7 (C-9); 182.1 (C-10); 118.3 (C-1a); 134.5 (C-4a); 132.5 (C-5a); 116.8 (C-8a); 21.8 (C-11); 100.5 (C-1′); 73.4 (C-2′); 76.6 (C-3′); 69.6 (C-4′); 77.3 (C-5′); 60.7 (C-6′).

### 2.4. α-glucosidase inhibitory activity assay

The α-glucosidase inhibitory activity was determined according to the described method [5]. A reaction mixture containing 500 μl of 67 mM phosphate buffer (pH 6.8), 20 μl of 0.3 U/ml fungal α-glucosidase in cold deionized water and 20 μl of sample was pre-incubated in
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athermoregulator for 5 minute at 37 °C, and then 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) solution was added to the mixture. After further incubation at 37 °C for 30 min, the reaction was stopped by adding 2440 µl of 100 mM Na₂CO₃ (pH 9.6). The released pNP was monitored spectrophotometrically by measuring UV absorbance at 400 nm. Tannic acid was used as positive control.

The percentage of α-glucosidase enzyme inhibition by the sample was calculated by the following formula:

\[ \% \text{ inhibition} = \left[ \frac{A_C - A_S}{A_C} \right] \times 100 \]

where \( A_C \) is the absorbance of the control and \( A_S \) is the absorbance of the tested sample.

### 3. RESULTS AND DISCUSSION

#### 3.1. Assay for α-glucosidase inhibitory activity

The yeast α-glucosidase is used to investigate the inhibitory activity of the crude extract and fractions. The α-glucosidase inhibitory activities of all extracts against α-glucosidase were determined using p-nitrophenyl-α-D-glucopyranoside (pNPG) as a substrate and these were compared with tannic acid (Table 1).

**Table 1.** The α-glucosidase inhibitory activity of Ruellia tuberosa.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>IC₅₀ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ethyl acetate fraction</td>
<td>4.73</td>
</tr>
<tr>
<td>2</td>
<td>Methanol fraction</td>
<td>8.27</td>
</tr>
<tr>
<td>3</td>
<td>crude ethanol</td>
<td>15.84</td>
</tr>
<tr>
<td>4</td>
<td>hexane fraction</td>
<td>46.76</td>
</tr>
<tr>
<td>5</td>
<td>tannic acid</td>
<td>7.66 (ng/mL)</td>
</tr>
</tbody>
</table>

All samples showed inhibitory activity against α-glucosidase enzyme significantly. IC₅₀ values of samples of plant extracts are higher than tannic acid because in the extract, pharmacological active compounds are not pure. Of which, the ethyl acetate fraction was the most potent, with IC₅₀ of 4.73 µg/ml.

#### 3.2. Structural elucidation

The EtOAc portion was repeatedly chromatographed on silica gel, Sephadex LH-20, and silica gel reversed-phase to afford rutin (1), syringaresinol (2), catechin (3) and pulmatin (4). These compounds were identified by comparison of their spectroscopic data with literature values (Fig. 1).
Compound 1 was obtained as a yellow powder. The $^1$H-NMR (CD$_3$OD, 500MHz) indicated the presence of a tetra-substituted benzene and a tri-substituted benzene at $\delta_H$ [6.22 (1H, s); 6.41 (1H, s); 6.90 (1H, d, 7.5 Hz); 7.65 (1H, d, 7.5 Hz) and 7.68 (1H, s)]. Two anomeric protons were observed at $\delta_H$ 5.13 (1H, d, 7.0 Hz, H-1') and $\delta_H$ 4.54 (1H, s, H-1''). Along with oxymethine protons at 3.28-3.83 ppm and a methyl group at $\delta_H$ 1.14 (3H, d, 6 Hz). Two singlet signals at $\delta_H$ 6.41 (1H, s); 6.22 (1H, s) were assigned to H-8 and H-6, respectively. Three proton signals of an ABX pattern at $\delta_H$ 7.68 (1H, s); 7.65 (1H, d, 7.5 Hz); 6.90 (1H, d, 7.5 Hz) were assigned to H-2', H-6' and H-5'.

In the $^{13}$C-NMR spectrum, 27 carbon signals were observed, of which 15 carbons belong to a flavonol skeleton. The $^1$H-NMR and $^{13}$C-NMR data proved that two monosaccharides were $\beta$-D-glucose and $\alpha$-L-rhamnose. In HMBC spectrum, there are correlations between rhamnose anomeric proton $\delta_H$ 4.54 (1H, s) with glucose C-6'' and glucose anomeric proton $\delta_H$ 5.13 (1H, d, 7.0 Hz) with C-3 of flavonol framework. From these evidences above, compound 1 was determined as rutin [14].

Compound 2 was isolated as a white prisms, m.p 175-176 °C. The ESI-MS spectrum showed a pseudomolecular ion [M-H]$^-$ at m/z 417.0, consistent with molecular formula C$_{22}$H$_{26}$O$_8$. The $^1$H-NMR spectrum (500 MHz, acetone-$d_6$) of 1 exhibited the resonance signals of two hydroxy proton at $\delta_H$ 7.16 (2H, s), four aromatic protons at $\delta_H$ 6.68 (4H, s), two oxymethine protons at $\delta_H$ 4.67 (2H, d, 4 Hz), two methine protons at $\delta_H$ 3.10 (2H, m), two oxymethylene protons at $\delta_H$ 4.24 (2H, d, 5.5 and 6.5 Hz) and 3.87 (2H, d, 3 Hz) and four methoxy groups at $\delta_H$ 3.82 (12H, s). The $^{13}$C-NMR together with DEPT spectra revealed 22 carbon signals including four methoxyl groups at $\delta_C$ 56.6, 12 aromatic carbons, two oxymethine at $\delta_C$ 86.7, two methine at $\delta_C$ 55.3 and two oxymethylene at $\delta_C$ 72.3. NMR data suggested that 2 possessed lignan skeleton. Two aromatic protons and two methoxy protons appeared only at $\delta_H$
Acknowledgements.

Ruellia tuberosa.
syringaresin IC50 of 4.73 µg/ml. From this fraction, by column chromatography glucosidase enzyme significantly. Among that, the ethyl acetate fraction is the most potent, with

6.68 and 3.82, respectively, proving that the structure of 2 was symmetry. Based on the NMR data and in comparison with reported literature, compound 2 is syringaresinol [15].

Compound 3 was isolated as a white needle. The 1H-NMR spectrum showed signals of a trisubstituted benzene at [δH 6.86 (1H, d, 2.0 Hz, H-2')] 6.79 (1H, d, 8.0 Hz, H-5') and 6.74 (1H, dd, 2.0 & 8.0 Hz, H-6')], a tetrasubstituted benzene at [δH 5.95 (1H, d, 2.5 Hz, H-8) and 5.88 (1H, d, 2.5 Hz, H-6)], two oxymethine groups at [δH 4.59 (1H, d, 8.0 Hz, H-2) and 4.01 (1H, m, H-3)], and 1 methylene δH 2.89 (1H, dd, 5.5 & 16.0 Hz, H-4a) and 2.55 (1H, dd, 8.5 and 16.0 Hz, H-4b). The 13C-NMR spectrum presented 15 signals including 12 aromatic, 2 oxymethine and 1 methylene carbons. From the above NMR data along with previous literature reports led to a conclusion that 3 was catechin. Moreover, specific rotation of 3 is [α]25 D+ 25.7 (c 0.01, methanol), chemical structure of 3 is (+)-catechin [16].

The 1H-NMR of 4 appeared two meta protons at δH 7.71 (1H, s) and 7.54 (1H, s); 3 protons of trisubstituted benzene at δH 7.76 (1H, t, 8.0 Hz); 7.67 (1H, d, 8.0 Hz) and 7.36 (1H, d, 8.0 Hz), together with a methyl group at δH 2.50 (3H, s) and an anemic proton atδH 5.17 (1H, d, 8.0 Hz) as well as oxymethine signals from 3 to 4 ppm. The 13C-NMR indicated 21 carbon signals of 12 aromatic carbons, 2 carbon carbonyl signals, 1 methyl carbon and 6 carbon signals of glucose. The beta configuration of glucose is determined through J-coupling of anomic proton. Moreover, the appearance of two carbonyl groups proves that 4 is an anthraquinone glycoside. The chelated proton signal at δH 12.94 (1H, s) indicated the presence of a hydroxyl group at C-8. The carbon chemical shift of two carbonyls at 187.6 and 182.1 ppm implied that C-1 and C-8 attached two hydroxyls. HMBC correlation between anonic protons with C-1 confirmed glucose moiety linked to C-1. MS spectrum showed a pseudomolecular ion [M+H]+ at m/z 416.91, consistent with molecular formula C25H32O8. The glucose moiety was confirmed through fragment m/z 254.3 [M-glucose]+. Based on the above evidence, compound 4 was determined as pulmatin [17].

4. CONCLUSIONS

Our findings indicate that Ruellia tuberosa L. extracts show inhibitory activity against α-glucosidase enzyme significantly. Among that, the ethyl acetate fraction is the most potent, with IC50 of 4.73 µg/ml. From this fraction, by column chromatography four compounds as rutin (1), syringaresinol (2), catechin (3) and pulmatin (4) have been isolated and identified. Their structures were elucidated by spectroscopic method including MS, 1D and 2D- NMR and comparison with the literature values. This is the first time these compounds were found in Ruellia tuberosa.

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