

Antioxidant and α -Glucosidase Inhibitory Activities of Isolated Compounds from *Ipomoea aquatica*

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Abstract: *Ipomoea aquatica* Forsk is a green leafy vegetable that is a rich source of amino acids and vitamins. Antioxidant and α -glucosidase inhibitory activities of the hexane (IAHE) and methanol (IAME) fractions of the vegetable portion of *I. aquatica* were investigated. The IAME fraction exhibited a strong scavenging effect of the 2,2-diphenyl-2-picryl hydrazyl (DPPH) free radicals, and this fraction contained the highest phenolic contents. Phytochemical investigation of the IAME fraction yielded three known compounds, namely 3,5-di-*O*-caffeoylquinic acid (**1**), 4,5-di-*O*-caffeoylquinic acid (**2**) and quercetin 3-*O*- β -D-glucoside (**3**). The structures were unambiguously elucidated based on 1D and 2D-NMR analyses (¹H, ¹³C, COSY, HSQC, HMBC) and mass spectrometry data. Compound **3** was isolated for the first time from this plant.

Keywords: Antioxidant activity; total phenolic content; α -glucosidase inhibition; spectral analysis. © 2016 ACG Publications. All rights reserved.

1. Introduction

Water spinach (*Ipomoea aquatica*, Forsk) is a member of the family Convolvulaceae and a well-known vegetable. This plant is native to southern Asia, and it is a fast-growing herbaceous vine that is commonly found creeping on muddy stream banks or floating in freshwater marshes and ponds. Because the plant has been cultivated for centuries, a large number of cultivars have been reported worldwide [1]. Previous studies on *I. aquatica* reported a diverse array of compounds such as myricetin, quercetin, luteolin, apigenin, and kaempferol, xanthophyll, taraxanthin, nicotinic acid, riboflavin, vitamin A, vitamin B1, vitamin C, vitamin E, and anthocyanins. The reported pharmacological activities include antidiabetic, antioxidant, anticancer and antiarthritic[2].

Diabetes mellitus is a metabolic disorder that is characterized by an abnormal postprandial elevated blood glucose level [3]. Among the different methods for controlling postprandial hyperglycemia, inhibition of α -glucosidase is considered as an effective approach. Mammalian α -glucosidase (α -D-glucoside glucohydrolase, EC 3.2.1.20) is the key enzyme that catalyzes the

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hydrolysis of carbohydrates, and this enzyme acts by retarding the liberation of glucose from oligosaccharides and disaccharides, reducing postprandial plasma glucose levels [4].

A previous phytochemical study on *I. aquatica* isolated isochlorogenic acids a, b and c, which exhibited inhibitory activity of disaccharide-degrading enzyme [5]. Our research aimed to identify more substances from the active fraction of *I. aquatica*. The phytochemical investigation of the IAME fraction has led to the isolation of two phenylpropanoids and a flavonoid namely, 3,5-di-*O*-caffeoylquinic acid (**1**), 4,5-di-*O*-caffeoylquinic acid (**2**) and quercetin-3-*O*- β -glucoside (**3**), respectively.

2. Materials and Methods

2.1. General Experimental procedures

Melting points of the isolated compounds were determined using Fisher-Johns aluminum heating stage melting points apparatus operational with adjustable magnifier mode. The NMR spectra were recorded on a Variance UNITY INOVA 500 MHz spectrometer measured at 500 MHz and 125 MHz, respectively. Chemical shifts (δ) were recorded in parts per million (ppm) relative to the tetramethyl silane (TMS) signal as an internal standard. The ESI-MS spectrum was recorded on a Thermo Finnigan model LCQ^{DECA} (San Jose, CA, USA) ion-trap mass spectrometer equipped with an ESI source coupled to a Surveyor HPLC binary pump, Surveyor diode array detector. Preparative HPLC analysis were carried out using a Jasco PU 2086 series pumping system equipped with Jasco MD-2010 PDA detector set to multiwavelength detection (218, 250 and 350) and Chromnav software. Column chromatography (CC) was performed using either silica gel Merck 7734 (70–230 mesh ASTM) or Merck 9385 (230–400 mesh ASTM) and Sigma Lipophilic Sephadex LH-20 (Fluka), and TLC by using pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck).

2.2 Plant Materials

The *I. aquatica* seeds were acquired from Green World Genetics, Malaysia. The seeds were planted in a field at the UPM Agricultural Park. A plot was established in an open field with temperature ranged from 22 - 32°C and the relative humidity ranged from 80 to 90% (Malaysian Meteorological Department, Ministry of Science, Technology and Innovation). The plants were exposed to direct sunlight and grown according to the protocol in 'Asian Vegetable Research & Development Centre' developed by [6].

2.3 Extraction and Isolation

The vegetative part (leaves and stem, 1.5 kg) was extracted with 70% methanol (3 L \times 3 times) at room temperature. The extracts were combined and concentrated under reduced pressure, and the resulting residue (65 g) was fractionated from water into n-hexane followed by methanol. The resulting fractions were evaporated to yield 7.6 g of n-hexane and 9 g of methanol soluble material. Eight grams of the methanol fraction were subjected to CC and eluted with chloroform and methanol with increasing polarities to obtain 15 major fractions (i.e., F₁₋₁₅). All of these fractions were screened for α -glucosidase inhibitory activity and antioxidant activity. Among these fractions, F₀₅₋₀₈ exhibited the highest biological activity, and therefore, these fractions were subjected to further purification. They were subjected to column chromatography over a silica gel (63 g; 9835; 3 cm x 20 cm) and eluted with chloroform and methanol, starting with a less polar gradient system to a more polar gradient system and finally methanol. Sub-fraction SE_{05-08/3} (0.12 g) was subjected to preparative

reverse phase HPLC on a XTerra MS C18 column using 70% methanol: water as the solvent system. Three pure compounds were collected; they were 3,5-di-*O*-caffeoylquinic acid (**1**) (2.8 mg, 0.023% yield), 4,5-di-*O*-caffeoylquinic acid (**2**) (3.1 mg, 0.025% yield) and quercetin 3-*O*- β -D-glucoside (**3**) (1.1 mg, 0.009% yield).

2.4 Spectroscopic Data of Compounds 1-3

2.4.1. 3,5-di-*O*-caffeoylquinic acid (**1**)

White powder, [9] UV (MeOH) λ_{\max} nm (log ϵ): 225, 247, 280, 350; ESI-MS at m/z 515 [M-H]⁻, (calculated for C₂₅H₂₄O₁₂), ¹H and ¹³C NMR data, see Table 1 in Supporting Information.

2.4.2. 4,5-di-*O*-caffeoylquinic acid (**2**)

White powder, [9] UV (MeOH) λ_{\max} nm (log ϵ): 225, 247, 280, 350; ESI-MS at m/z 515 [M-H]⁻, (calculated for C₂₅H₂₄O₁₂), ¹H and ¹³C NMR data, see Table 1 in Supporting Information.

2.4.3. Quercetin 3-*O*- β -D-glucoside (**3**)

A yellow powder, [10] UV (MeOH) λ_{\max} nm (log ϵ): 356 (4.05), 297 (3.83), 256 (4.13). ESI-MS at m/z 463 [M-H]⁻, (Calculated for C₂₁H₂₀O₁₂), ¹H and ¹³C NMR data, see Table 2 in Supporting Information.

2.5. α -Glucosidase inhibition assay

The α -glucosidase inhibitory assay was performed in a 96-well microtiter plate according to [7] with some modifications. Briefly, 10 μ L of the test compound in 0.5% DMSO was subsequently diluted with 100 μ L (0.03 M phosphate buffer pH 6.5) then mixed with 15 μ L (0.2 U/mL) of enzyme solution and incubated for 10 min at room temperature. After pre-incubation 75 μ L of the substrate *p*-nitrophenyl α -D-glucopyranoside (0.5 mM concentration in 0.05 M phosphate buffer, pH 6.5) was added to the mixture, and allowed to incubate at room temperature for 15 min, and then the reaction was terminated by the addition of 50 μ L glycine solution (2M, pH 10). The optical density (OD) was measured by spectrophotometry at 405 nm. Percent inhibition of the enzyme was calculated in comparison with the control and expressed as mean \pm SD.

2.6. DPPH radical scavenging assay (RSA)

The antioxidant potential of the cultivars were estimated by radical scavenging effect using a DPPH free radical scavenger assay according to [8]. Butylated hydroxytoluene (BHT) was used as reference compound. Briefly, different (50 μ L) dilutions (18 - 300 μ M) of the isolates and 100 μ L DPPH (0.2 mM in methanol) were added as the free radical source, and incubated for 30 min. Optical density (OD) of the solution was read using an ELISA Reader (SPECTRA max PLUS, USA) at 517 nm. A methanolic solution of DPPH served as negative control. All assays were performed in three replicates. IC₅₀ values denote the concentration of sample required to scavenge 50% DPPH free radicals. Percent inhibition was calculated using the following formula:

$$\text{Inhibition \%} = [(A \text{ control} - A \text{ sample}) / A \text{ control}] * 100\%$$

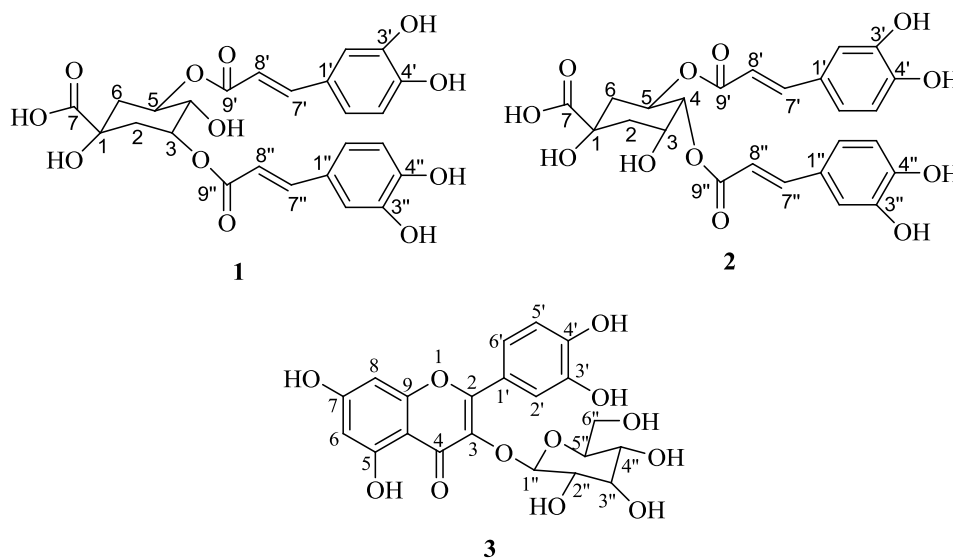


Figure 1. Structures of compounds isolated from *I. aquatica* Forsk.

3. Results and Discussion

3.1. Structure elucidation

Compound **(1)** was obtained as a white amorphous powder. The molecular ion peak of **(1)** was located at m/z 515 $[M-H]^-$ in the ESI-MS (negative) spectrum, indicating that the molecular weight of **(1)** was 516 corresponding to a molecular formula of $C_{25}H_{24}O_{12}$. Its UV spectrum exhibited a maximum absorption at 245 and 320 nm indicating the presence of caffeic acid moiety. The 1H NMR spectrum of **(1)** (Table 1) indicated the presence of two tri-substituted aromatic ring protons at δ 7.07 (1H, br. s, H-2'' and H-2'), 6.78 (1H, d, $J = 8.0$ Hz, H-5''), 6.96 (1H, br. d, $J = 8.0$ Hz, H-6''), 6.78 (1H, d, $J = 8.0$ Hz, H-5'), and 6.98 (1H, br. d, $J = 8.0$ Hz, H-6') and four protons in trans-configuration for double bonds at δ 7.57 (1H, d, $J = 15.5$ Hz, H-7'), 6.28 (1H, d, $J = 15.5$ Hz, H-8'), 7.63 (1H, d, $J = 15.5$ Hz, H-7''), and 6.39 (1H, d, $J = 15.5$ Hz, H-8''). Additionally, the 1H NMR spectrum of **(1)** indicated the presence of a quinic acid moiety, including four methylene protons [δ 2.33 (1H, d, $J = 14.0$ Hz, H-2ex), 2.27 (2H, dd, $J = 14.5, 7$ Hz, H-6eq), 2.24 (1H, m, H-6ax), 2.21 (1H, m, H-6eq)] and three oxygenated methine protons [δ 5.45 (1H, m, H-3), 3.97 (1H, dd, $J = 7.5, 3.0$ Hz, H-4), 5.38 (1H, m, H-5)]. Moreover, the presence of two trans-caffeoyl groups was indicated by the observation of two ABX systems and two trans-olefinic protons. The ^{13}C -NMR spectrum of **(1)** exhibited resonances for twenty five (25) carbons, with twelve (12) being aromatic carbons resonating from δ 113.6 to 167.3. Eight (8) of the aromatic carbons signals resonating at δ 126.4, 126.1, 145.7, 145.9, 148.8, 149.0, 166.8 and 167.3 were assigned to six quaternary carbons in the two caffeic acid moieties at C-1', C-1'', C-3', C-3'', C-4', C-4'', C-9' and C-9''. Four methine carbons signals located at δ 114.8, 121.9, 145.9 and 113.9 were assigned to C-5', C-6', and C-7' and C-8' for each of the caffeic groups. In addition, three oxymethine resonances at δ 71.6, 70.7 and 70.5 were assigned C-3, C-5 and C-4, and two sp^3 methylenes were located at δ 37.1 and 35.2; an oxygenated quaternary carbon at C-1 73.6, and a carboxyl resonance at C-7 178.2. All of these resonances were characteristic of quinic acid. These assignments were also supported by the COSY, HSQC and HMBC correlations. Therefore, based on these spectral data, the structure of compound **(1)** was unambiguously identified as known 3,5-di-O-caffeoylquinic acid [9].

Compound **(2)** was obtained as a white amorphous powder. The molecular ion peak of **(2)** was revealed at m/z 515 $[M-H]^-$ in the ESI-MS spectrum, indicating that the molecular weight of **(2)**

was 516, corresponding to a molecular formula of $C_{25}H_{24}O_{12}$. Its UV spectrum exhibited absorption maximas at 245 and 320 nm indicating the presence of a caffeic acid moiety. The 1H NMR spectrum of compound (2) exhibited one quinic acid and two caffeoyl moieties similar to those in compound (1), and the only difference is in the attachments of the two caffeoyl moieties at C-4 and C-5 of the quinic acid part, which were deduced from the HMBC correlations of H-4 and H-5, respectively, with their ester carbonyl carbons. In addition, downfield shifts for H-4 by -1.14 ppm and H-5 by -0.27 ppm were observed, downfield from those of quinic acid, respectively. Therefore, the substitution attachments of the two caffeoyl units were located at carbons 4 and 5. The comparison of the spectroscopic data with the literature also confirmed the proposed structure. Therefore, based on these spectral data, the structure of compound (2) was identified as known 4,5-di-*O*-caffeoylquinic acid) [9].

Compound (3) is a yellow amorphous solid, with a melting point of 210-216 °C. The UV spectrum of (3) exhibited a strong absorption at 350nm and 250 nm, which is characteristic of flavonols. In the ESI-MS of this compound the molecular ion peak appeared at m/z 463 (M-H)⁻ which corresponds to a molecular formula of $C_{21}H_{20}O_{12}$ with an aglycon of the flavonoids at m/z 301 (M-H)⁻ (glucose). The 1H -NMR of (3) exhibited doublets at δ 7.71 (1H, d, $J = 2.0$ Hz), which were assigned to proton H-2' of the B-ring. In addition, proton H-6' appears as a doublet at 7.59 ($J = 8.5, 2.0$ Hz). This proton is meta coupled ($J = 8.5$ Hz) to proton H-2' and ortho coupled ($J = 2.0$ Hz) to proton H-5' at 6.21 (d, 1H, $J = 1.5$ Hz) and 6.40 (d, 1H, $J = 1.5$ Hz) due to the protons at C-6 and C-8, respectively. These protons, which are located on the A ring of the quercetin skeleton, have low J values because they are meta-coupled to each other. Furthermore, signals at δ 3.3 – 4.2 as well as a doublet 0.94 with a J value of 6.0 Hz were also observed. Based on the 2D experiments, the doublets at 5.27 with a J -value of 7.5 Hz was coupled to a proton at δ 3.4, indicating that the signal at δ 5.27 was due to an anomeric proton with a β -type linkage. The ^{13}C -spectrum indicated the presence of 21 carbon atoms, including one set of carbons in a glucose moiety. The resonance of six quaternary carbons were observed at δ_c 157.1, 134.1, 161.6, 164.8, 156.9 and 104.2 corresponding to positions C-2, C-3, C-5, C-7, C-9 and C-10 on the A-ring; 144.5 corresponding to position C-3 on the C-ring; and 148.4 and 121.4, corresponding to positions C-4' and C-1' on the B-ring. In addition, the spectrum suggested the presence of a carbonyl group resonating at δ 177.9, which was assigned to position C-4. The signals observed at δ 102.9, 76.9, 74.3, 69.2, 61.6, and 60.9 were assigned to positions C-1", C-3", C-5", C-2", C-4", and C-6", respectively, due to the glycosyl moiety. These assignments were also supported by the HSQC and HMBC correlations. By comparison to previously reported literature data [10], this compound is quercetin-3-*O*- β -glucoside, and it was not isolated from this plant before.

3.2 Antioxidant and antidiabetic activity

Compounds (1) and (2) exhibited good antioxidant activity with IC_{50} values of 9.4 ± 0.06 μM and 12.9 ± 0.12 μM , respectively, and compound (3) exhibited the lowest activity with IC_{50} values of 16.5 ± 0.19 μM compared to standard BHT; 19.3 ± 0.15 μM . The presence of a catechol moiety in the structure is primarily responsible for the antioxidant activity, and the additional presence of hydroxyl groups in the compounds are responsible for the maximum radical scavenging capability [11]. Moreover, these compounds also exhibited potent α -glucosidase inhibitory activity and the concentrations of each compound for 50% inhibition of the enzymatic activity (IC_{50}) are shown in Table 1. Compounds (1) and (2) were the most potent active compounds identified in this study with IC_{50} values of 18.7 ± 0.17 μM and 14.5 ± 0.19 μM , respectively, and compound (3) exhibited lower activity with IC_{50} value of 20.5 ± 0.21 μM compared to the positive control quercetin with IC_{50} value of 16.2 ± 0.023 μM . To the best of our knowledge, this is the first report of the α -glucosidase inhibitory activity of compound (3) in *I. aquatica*. Previously, compounds (1) and (2) have been reported to exhibit α -glucosidase inhibitory activity [5]. The unsaturated C-ring, 4-CO, 3-OH, the linkage of the B-ring at position 3, and the hydroxyl substitution on the B-ring increased the α -glucosidase inhibitory

activity [12]. Subsequently, the inhibition kinetics of α -glucosidase by compound (2) was analyzed using Lineweaver–Burk and Dixon plots. The type of inhibition was deduced from the calculation of K_m (Michaelis-Menten constant) and V_{max} (maximum enzyme velocity) parameters obtained by the nonlinear regression Michaelis-Menten enzyme kinetics which was complemented by the construction of the Lineweaver-Burk double reciprocal plots. In the absence of inhibitors, a K_m value of 278 μM and a V_{max} value of 32.5 $\mu\text{mol}/\text{min}^{-1}$ were observed. As illustrated in Fig. 2, the inhibition kinetics analyzed by Dixon plots show that compound (2) is a noncompetitive inhibitor because increasing substrates resulted in a series of lines with a common intercept near the x-axis but with different slopes. The inhibition kinetics analyzed by Lineweaver–Burk plots also confirmed that compound (2) is a noncompetitive inhibitor.

Table 1. Antioxidant and α -glucosidase inhibitory activities of isolated compounds from *I. aquatica*. Forsk.

Compounds	DPPH IC_{50} , μM	α -Glucosidase inhibition IC_{50} , μM
1	9.4 \pm 0.06	18.7 \pm 0.17
2	12.9 \pm 0.12	14.5 \pm 0.19
3	16.5 \pm 0.19	20.5 \pm 0.21
BHT	19.3 \pm 0.15	
Quercetin		16.2 \pm 0.023

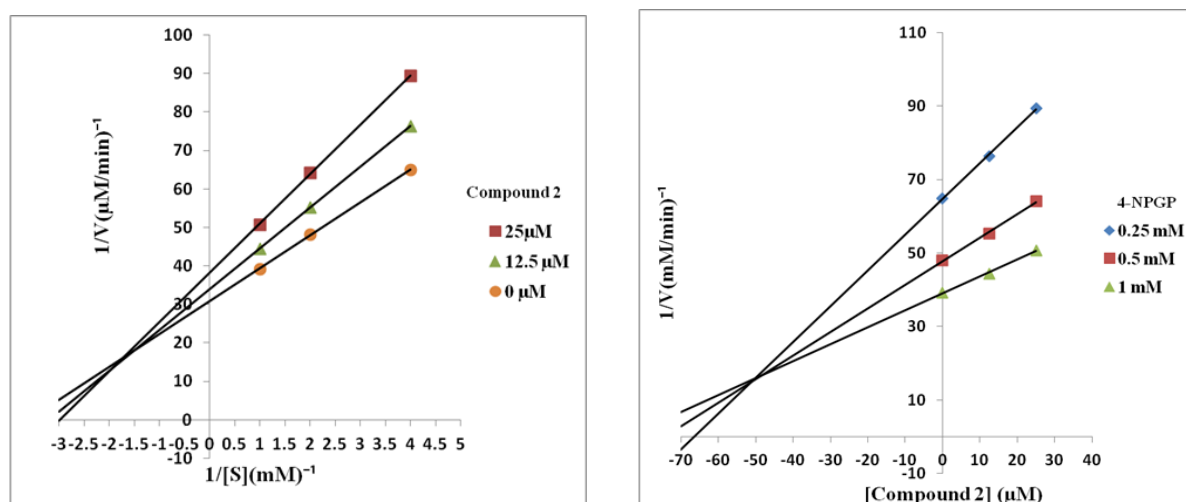


Figure 2. Lineweaver–Burk (a) and Dixon (b) plots of α -glucosidase inhibition at different concentrations of substrate and compound 2

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Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/RNP>

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