Antioxidant, α-glucosidase inhibitory activities, and HPLC quantitative analysis of phenolic compounds isolated from *Neptunia oleracea* Lour.

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Abstract

Neptunia oleracea Lour. is a tropical plant cultivated in Southeast Asia. It is consumed as vegetable and traditional herb for the treatment of several disorders. The objective of the present work was to isolate the phenolic compounds from *N. oleracea*, followed by their bioactivity evaluation and quantitative analysis. The ethyl acetate (EtOAc) and methanol (MeOH) fractions of *N. oleracea* were subjected to various chromatographic techniques to isolate the phenolic compounds. The isolated phenolic compounds were characterised by several spectroscopic methods, including mass spectrometry (MS) and nuclear magnetic resonance (NMR). Then, these compounds were subjected to DPPH free radical scavenging as α-glucosidase inhibitory assays for the evaluation of their activities. Their contents in the fractions were analysed via high performance liquid chromatography (HPLC) quantitative analysis. Five phenolic compounds including quercetin-3-*O*-β-D-xylopyranoside (**1**), quercetin-3-*O*-α-L-arabinopyranoside (**2**), quercetin-3-*O*-α-L-rhamnoside (**3**), methylgallate (**4**) and rutin (**5**) were isolated from *N. oleracea* for the first time. Evaluation on the DPPH free radical scavenging and α-glucosidase inhibitory activities of these compounds showed that methylgallate (**4**) was the most potent antioxidant and α-glucosidase inhibitors among them, with IC_{50} values of 17.25 and 50.76 µM, respectively. The HPLC quantitative analysis revealed the high content of the quercetin derivatives (compounds **1**, **2** and **3**) in the EtOAc fraction (ranging from 125.68 to 157.55 µg/mg) and methylgallate (**4**) in the MeOH fraction (75.25 µg/mg). Comparison of the bioactivities of the isolated phenolic compounds with the fractions indicated their significant contribution for the DPPH free radical scavenging of *N. oleracea*; while they might be working synergistically for the α-glucosidase inhibitory activity. The results of the present work could help to validate the contribution of phenolic compounds for the studied bioactivities of *N. oleracea*.

Introduction

Diabetes mellitus is a chronic disease in which the patient experiences a high level of blood glucose due to the insufficient production or ineffective usage of insulin by the body. It has affected approximately 9% of adults globally, and is predicted to become the seventh leading cause of death by 2030 (WHO, 2016). The currently available synthetic drugs, such as metformin and acarbose meet few of the needs of diabetic patients, and cause undesirable side effects (Hung *et al.*, 2012; Hasanein and Mohammad Zaheri, 2014). On the other hand, accumulated evidence reveals that isolated compounds, enriched fractions and extracts from natural sources may possess antioxidant and α-glucosidase inhibitory properties which can help to manage diabetes mellitus and its associated complications (Yao *et al.*, 2010; Hung *et*

al., 2012; Zhang *et al.*, 2013; Indariani *et al.*, 2014). Hence, natural resources including fruits, vegetables and traditional medicinal plants have drawn the attention of researchers worldwide.

Neptunia oleracea Lour. (Synonym: *N. prostrata* Lamk.) is an aquatic plant widely distributed over the tropical regions of the world. It is cultivated in rural areas, especially those in Southeast Asia. It is consumed as vegetable as well as traditional herb for the treatment of several ailments such as fever, earache, poisoning, constipation and gastritis (Paul *et al.*, 2012; Deb *et al.*, 2013). This plant has been reported to possess potential antiinflammatory, antiulcer, antimicrobial and anticancer properties (Nakamura *et al.*, 1996; Bhoomannavar *et al.*, 2011a; Bhoomannavar *et al.*, 2011b). Besides, results from our previous work also revealed the potency of this plant in DPPH free radical scavenging and α-glucosidase inhibitory activities (Lee *et al.*, 2014). Phenolic compounds including derivatives of quercetin, kaempferol and apigenin as well as phenolic acids have been revealed as the important free radical scavengers and α-glucosidase inhibitors in *N. oleracea* (Lee *et al.*, 2016; Lee *et al.*, 2018).

In view of the importance of phenolic compounds in contributing to the DPPH free radical scavenging and α-glucosidase inhibitory activities of *N. oleracea*, the present work attempts to isolate these compounds using various chromatographic procedures. The isolated phenolic compounds were characterised and individually tested for their activities. Their contents in the fractions were also analysed using quantitative high performance liquid chromatography (HPLC). The results of the present work may show the significance of the phenolic compounds as the phytochemical markers of *N. oleracea* for the DPPH free radical scavenging and α-glucosidase inhibitory activities. The work on the isolation, biological activity evaluation and quantitative analysis of the phenolic compounds were reported for the first time for *N. oleracea*.

Materials and methods

General

Solvents used in all the chromatography included *n*-hexane, chloroform, ethyl acetate (EtOAc), methanol (MeOH) and water. Lichroprep RP-18 (Merck) and C_{18} Isolute® SPE cartridge (Biotage, Mid Glamorgan, UK) were used for column chromatography. Thin layer chromatography (TLC) was routinely used to detect and monitor the presence of compounds in the fractions as well as in the subsequent subfractions. Spots were visualised using

10% sulfuric acid in ethanol and heating. Preparative HPLC was performed using Xterra® Perp MS C_{18} (19 mm × 150 mm, 5 μm; Waters, Milford, MA, USA) preparative column on a JASCO liquid chromatography system equipped with JASCO PU-2086 Plus Intelligent preparative pumps and JASCO UV-2077 Plus 4-wavelength Intelligent UV/VIS detector. The ^{1}H and ^{13}C NMR as well as the gradient homonuclear correlation spectroscopy (gCOSY), adiabatic gradient heteronuclear single quantum correlation (gHSQCAD) and heteronuclear multiple bond coherence (gHMBCAD) experiments of every isolate were acquired using a 500 MHz Varian INOVA NMR spectrometer. Chemical shifts were recorded as part per million (ppm) relative to tetramethyl silane (TMS).

Plant material

Neptunia oleracea was identified by Dr. Shamsul Khamis, an in-house botanist of the Institute of Bioscience, (UPM), and the voucher specimen (SK 2516/14) has been deposited in the Herbarium of the institute. The plant was then planted by distributing the stem to a corner of a pond located in UPM Agricultural Park. Bamboo was used to fence the corner to prevent the plant from invading to the centre of the pond, which will make the harvest harder. The plant was harvested after three months of planting.

Extraction, fractionation and isolation

The freeze dried and powdered *N. oleracea* leaves (40 g) were extracted with 1 L of absolute ethanol via sonication (1 h, at controlled temperature). From this, 10 g ethanolic crude extract was produced. To yield fractions with different polarities, the obtained crude extract was subjected to solid phase extraction (SPE). The crude extract (1 g, premixed with 2 g of silica) was loaded onto hexane-activated and conditioned 10 g silica Isolute® cartridge (Biotage, Mid Glamorgan, UK) and eluted successively with hexane, chloroform, EtOAc and MeOH to yield the respective fractions. Each solvent was allowed to pass through the cartridge until the eluent obtained was colourless. From our preliminary results, the DPPH free radical scavenging and α-glucosidase inhibitory activities reside mainly in the EtOAc and MeOH fractions. The strong correlation was observed between phenolic compounds and the activities of the EtOAc and MeOH fractions. Hence, EtOAc and MeOH fractions were selected for the isolation of phenolic compounds.

The EtOAc fraction (700 mg) was further fractionated by SPE (20 g C_{18} adsorbent) using water and MeOH (ratio 6:4) as eluent. A total of

14 subfractions (E$_{1-14}$) were obtained. Based on the TLC results, flavonoids were mainly distributed in subfractions E$_3$ to E$_7$ (observed as yellow spots after heating with the visualisation reagent), with highest intensities in subfraction E$_4$. In addition, the HPLC profiles of these subfractions also showed that subfraction E$_4$ had the highest intensities of the targeted peaks. Consequently, subfraction E$_4$ (65 mg) was subjected to preparative HPLC using 0.1% acetic acid in water (solvent A) and acetonitrile (solvent B) as the mobile phase. Isocratic elution at solvent composition of 80% A and 20% B over 60 minutes at a flow rate of 4.0 mL/min yielded **1** (1.5 mg), **2** (1.5 mg) and **3** (1 mg).

The MeOH fraction (1 g) was chromatographed over an open glass column (2.5 cm diameter x 27 cm height) packed with Lichroprep RP-18, with a gradient of water-MeOH (from 6:4 to 0:1, v/v) as an eluent. Altogether, 22 subfractions (M$_{1-22}$) were obtained. Less intense yellow spots were observed in the TLC of these subfractions. Hence, the subfractions with high intensity of major TLC spots (M$_2$ and M$_8$) were selected for further isolation in order to increase the possibility of getting pure compound. Subfractions M$_2$ (182 mg) and M$_8$ (20 mg) were subjected to preparative HPLC, with 0.1% acetic acid in water (A) and acetonitrile (B) at a flow rate of 4.0 mL/min as eluent. The elution of subfraction M$_2$ began with 95% solvent A for 10 min, followed by lowering of A to 75% over 50 min and this afforded **4** (1.3 mg). Elution of subfraction M$_8$ using isocratic system with 80% A afforded **5** (1.2 mg). All isolated compounds were characterised and identified via spectroscopic methods and comparison with published data. The physical and spectral data of the isolated phenolic compounds were as follow:

Quercetin-3-O-β-D-xylopyranoside **(1)**. Light yellow amorphous powder; ESI-MS at m/z 433 [M-H]$^-$: m/z 301, 300, 283, 271, 255, 179, 151; ^{1}H NMR (Acetone-d_6, 500 MHz): δ 6.27 (1H, d, J = 2.0 Hz, H-6), 6.51 (1H, d, J = 2.0 Hz, H-8), 7.78 (1H, d, J = 2.0 Hz, H-2'), 6.95 (1H, d, J = 8.5 Hz, H-5'), 7.66 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 5.33 (1H, d, J = 6.5 Hz, H-1"), 3.55 (2H, m, H-2", H-4"), 3.49 (1H, m, H-3"), 3.17 (1H, dd, J = 11.5, 8.5 Hz, H-5"a), 3.81 (1H, dd, J = 11.5, 4.5 Hz, H-5"b); ^{13}C NMR (Acetone-d_6, 125 MHz): δ 157.1 (C-2), 134.6 (C-3), 178.4 (C-4), 162.2 (C-5), 98.8 (C-6), 164.5 (C-7), 93.7 (C-8), 157.1 (C-9), 104.7 (C-10), 121.9 (C-1'), 116.2 (C-2'), 144.7 (C-3'), 148.5 (C-4'), 115.0 (C-5'), 122.1 (C-6'), 103.4 (C-1"), 73.6 (C-2"), 75.9 (C-3"), 69.6 (C-4"), 65.7 (C-5").

Quercetin-3-O-α-L-arabinopyranoside **(2)**. Light yellow amorphous powder; ESI-MS at m/z 433 [M-H]$^-$: m/z 301, 300, 271, 255, 179, 151; ^{1}H NMR (Acetone-d_6, 500 MHz): δ 6.27 (1H, d, J = 2.0 Hz, H-6), 6.51 (1H, d, J = 2.0 Hz, H-8), 7.85 (1H, d, J = 2.0 Hz, H-2'), 6.95 (1H, d, J = 8.5 Hz, H-5'), 7.67 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 5.28 (1H, d, J = 6.0 Hz, H-1"), 3.93 (1H, m, H-2"), 3.72 (1H, dd, J = 7.0, 3.5 Hz, H-3"), 3.85 (2H, m, H-4", H-5"b), 3.44 (1H, dd, J = 12.0, 2.5 Hz, H-5"a); ^{13}C NMR (Acetone-d_6, 125 MHz): δ 157.0 (C-2), 134.5 (C-3), 178.2 (C-4), 162.0 (C-5), 98.7 (C-6), 164.4 (C-7), 93.7 (C-8), 157.0 (C-9), 104.6 (C-10), 121.8 (C-1'), 116.4 (C-2'), 144.8 (C-3'), 148.7 (C-4'), 115.0 (C-5'), 122.0 (C-6'), 103.2 (C-1"), 71.5 (C-2"), 72.8 (C-3"), 67.1 (C-4"), 65.1 (C-5").

Quercetin-3-O-α-L-rhamnoside **(3)**. Light yellow crystals; ESI-MS at m/z 447 [M-H]$^-$: m/z 301, 300, 271, 255, 179, 151; ^{1}H NMR (CD$_3$OD, 500 MHz): δ 6.20 (1H, d, J = 2.0 Hz, H-6), 6.37 (1H, d, J = 2.0 Hz, H-8), 7.33 (1H, d, J = 2.0 Hz, H-2'), 6.90 (1H, d, J = 8.5 Hz, H-5'), 7.31 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 5.34 (1H, d, J = 1.5 Hz, H-1"), 4.21 (1H, dd, J = 3.0, 1.5 Hz, H-2"), 3.74 (1H, dd, J = 9.5, 3.5 Hz, H-3"), 3.33 (1H, m, H-4"), 3.43 (1H, m, H-5"), 0.93 (3H, d, J = 6.5 Hz, H-6"); ^{13}C NMR (CD$_3$OD, 125 MHz): δ 157.9 (C-2), 134.8 (C-3), 178.2 (C-4), 161.8 (C-5), 98.4 (C-6), 164.6 (C-7), 93.3 (C-8), 157.1 (C-9), 104.4 (C-10), 121.5 (C-1'), 115.5 (C-2'), 145. (C-3'), 148.5 (C-4'), 114.9 (C-5'), 121.4 (C-6'), 102.2 (C-1"), 70.5 (C-2"), 70.6 (C-3"), 70.7 (C-4"), 71.8 (C-5"), 16.3 (C-6").

Methylgallate **(4)**. White needle crystals; ^{1}H NMR (CD$_3$OD, 500 MHz): δ 7.03 (2H, s, H-2, H-6), 3.81 (3H, s, H-8); ^{13}C NMR (CD$_3$OD, 125 MHz): δ 121.8 (C-2), 110.3 (C-2), 146.6 (C-3), 139.8 (C-4), 146.6 (C-5), 110.3 (C-6), 169.1 (C-7), 52.4 (C-8).

Rutin **(5)**. Yellow amorphous powder; ESI-MS at m/z 609 [M-H]$^-$: m/z 301, 300, 271, 179, 151; ^{1}H NMR (CD$_3$OD, 500 MHz): δ 6.21 (1H, s, H-6), 6.40 (1H, s, H-8), 7.66 (1H, d, J = 2.0 Hz, H-2'), 6.87 (1H, d, J = 8.0 Hz, H-5'), 7.63 (1H, dd, J = 8.0, 2.0 Hz, H-6'), 5.10 (1H, d, J = 7.5 Hz, H-1"), 4.51 (1H, s, H-1'''), 1.11 (3H, d, J = 6.5 Hz, H-6''') ; ^{13}C NMR (CD$_3$OD, 125 MHz): δ 157.9 (C-2), 134.2 (C-3), 178.1 (C-4), 161.6 (C-5), 98.5 (C-6), 164.6 (C-7), 93.4 (C-8), 157.1 (C-9), 104.3 (C-10), 121.7 (C-1'), 116.2 (C-2'), 144.4 (C-3'), 148.4 (C-4'), 114.7 (C-5'), 122.1 (C-6'), 103.3 (C-1"), 74.3 (C-2"), 75.8 (C-3"), 70.0 (C-4"), 76.7 (C-5"), 67.1 (C-6"), 101.0 (C-1'''), 70.7 (C-2'''), 70.8 (C-3'''), 72.5 (C-4'''), 68.3 (C-5'''), 16.5 (C-6''').

DPPH free radical scavenging assay

The ability to scavenge DPPH free radical was performed according to the procedure previously described (Lee *et al.*, 2018). Briefly, the isolated phenolic compounds and quercetin (as positive control) were dissolved in DMSO and diluted with the same solvent to reach final concentrations required for the IC_{50} determination. In a 96-wellplate, 50 µL of test samples was mixed with 100 µL of DPPH (5.9 mg/100 mL MeOH). This was followed by incubation of the plate in the dark for 30 min. The absorbance was then measured at 517 nm using a micro-plate reader (SPECTRAmax PLUS, Sunnyvale, CA, USA). The percentage of scavenging (SC %) was calculated as SC % = $[(A_o - A_s)/A_o] \times 100$, where A_o refers to the absorbance of the reagent blank, and A_s refers to the absorbance of the tested samples. Three determinations were performed for every sample, and the results were expressed as IC_{50} values in µg/mL, which denote the concentration of sample needed to scavenge 50% of the DPPH free radicals.

α-Glucosidase inhibition assay

The α-glucosidase inhibition assay was performed as previously reported (Lee *et al.*, 2018). Briefly, the isolated phenolic compounds and quercetin (as positive control) were dissolved in DMSO and further diluted using DMSO containing buffer to obtain a series of concentrations required for the IC_{50} determination. The final concentration of DMSO in each well was 0.5%. The α-glucosidase enzyme and PNPG substrate were prepared in 50 mM phosphate buffer (pH 6.5). In each well, 10 µL of sample was mixed with 130 µL of 30 mM phosphate buffer (pH 6.5) and 10 µL of enzyme (0.02 U/well). Following 5 min pre-incubation, 50 µL of 1 mM PNPG substrate was added into each well, followed by further incubation for 15 min. The absorbance was measured at 405 nm immediately after adding 50 µL of glycine (pH 10) to stop the reaction. The percentage of inhibition (% inhibit) was calculated as % inhibit = $[(A_n - A_s)/A_n] \times 100\%$, where A_n refers to the absorbance of negative control, and A_s refers to the absorbance of tested samples. The analysis was performed in three determinations for every sample. The results were expressed as IC_{50} value in µg/mL.

Quantitative analysis of phenolic compounds isolated from bioactive fractions of Neptunia oleracea

Quantitative analysis of the isolated phenolic compounds in their respective fractions was carried out via HPLC analysis using the method of Mediani *et al.* (2015) with modifications. The samples of EtOAc

and MeOH fractions were prepared at 300 and 500 µg/mL, respectively. Meanwhile, the isolated phenolic compounds were used as standards and prepared by premixing them together to obtain the concentration of 20 µg/mL each (except rutin at 10 µg/mL). All the fractions and standards were prepared in HPLC-grade MeOH, and filtered through 0.45 µm nylon membrane before injection to the HPLC system. The Ultimate 3000 series system (Dionex, Idtsein, Germany) fitted with dual low-pressure gradient pump and DAD detector was employed. Separation of the analytes was done by Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2) column (4.6 mm × 250 mm, 5 µm), with 10 mmol methanesulfonic acid (solvent A) and acetonitrile (solvent B) as the mobile phase flowing at 1.0 mL/min under a gradient program. The gradient system was as follow: 5% B (1 min), 5-20% B (2 min, linear gradient), 20-40% B (20 min, gradient curve 7), 40% B (2 min), 40-100% B (2 min, linear gradient), 100% B (4 min), 100-5% B (0.5 min, linear gradient), and 5% B (2.5 min). The total analysis time per injection was 34 min. The injection volume for the samples was 20 µL. The wavelength used for identification of the phenolic compounds to be quantified with the DAD detector was 254 nm. The peaks of the phenolic compounds were confirmed by comparison of the retention times with those of the isolated ones. To quantitate them, calibration curves were constructed using the standard solution of isolated phenolic compounds injected at three different volumes (5, 10 and 20 µL). The calibration curves were accepted if the $R^2 \geq 0.99$. Triplicate injections were performed for each of the samples as well as the standard solution. The amount of the isolated phenolic compounds in their respective fraction was expressed as µg/mg of fraction.

Results and discussion

Characterisation of isolated compounds 1-5

Compound **1** appeared as light yellow amorphous powder. The ESI-MS spectrum of this compound in the negative ion mode showed the deprotonated molecular ion peak at m/z 433 and aglycone ion at m/z 300, which corresponded to a molecular formula of $C_{20}H_{18}O_{11}$ for this present compound, and $C_{15}H_{10}O_7$ for the aglycone (Figure S1). These data suggested the quercetin skeleton as the structure of this compound (Li *et al.*, 2016). The ^{1}H NMR spectrum of compound **1** showed the typical profile of quercetin with a sugar substitution, by comparing to reported characterisation (Park *et al.*, 2012). All the aromatic signals were attributed to quercetin. Both signals at δ 6.27 and δ 6.51 appeared as doublets with *J* value

of 2.0 Hz. This showed that they were meta coupled to one another, and hence were assigned to proton H-6 and H-8 on the ring A. Meanwhile, the other three aromatic signals at δ 7.78 (d, *J* = 2.0 Hz), δ 7.66 (dd, *J* = 8.5, 2.0 Hz) and 6.95 (d, *J* = 8.5 Hz) were assigned as H-2', H-6' and H-5', respectively on the ring B. The signal of proton H-6' appeared as a doublet of doublets due to its meta (*J* = 2.0 Hz) and ortho (*J* = 8.5 Hz) couplings with protons H-2' and H-5', respectively. Besides, the anomeric proton of the sugar (H-1") showed signal at δ 5.33 as a doublet with *J* value of 6.5 Hz. This indicated β-configuration of the sugar unit (Rao *et al.*, 1998; Park *et al.*, 2012). The other signals of the sugar unit resonated between δ 3.17 and 3.81.

The ^{13}C NMR spectrum of compound **1** showed the presence of 20 carbons. The signals attributed to the quercetin aglycone resonated between δ 178.1 and δ 98.7, with the carbonyl carbon (C-4) appeared at the most downfield. After deducting the number of carbon possessed by the quercetin aglycone, the ^{13}C NMR spectrum disclosed the presence of a five-carbon sugar. The anomeric carbon of the sugar appeared at δ 103.4; while the other sugar signals resonated between δ 75.9 and δ 65.6. Comparison of the present ^{1}H and ^{13}C spectral data with those reported previously identified the sugar unit as β-D-xylopyranose (Park *et al.*, 2012). Besides, the position of the β-D-xylose was confirmed on the C-3 of quercetin by the correlation observed between H-1" and C-3 in the gHMBCAD spectrum (Figure S2). Hence, based on these observations, compound **1** was characterised as quercetin-3-*O*-β-D-xylopyranoside.

Compound **2** was obtained as light yellow amorphous powder. The negative ion ESI-MS spectrum of this compound revealed the deprotonated molecular ion peak at m/z 433 as well as the aglycone ion at m/z 300, which were similar to compound **1** (Figure S3). Hence, this suggested that this compound might be the epimer of compound **1**. The ^{1}H and ^{13}C NMR spectra of compound **2** also revealed the skeleton of quercetin with single sugar moiety. All the signals in the aromatic region were contributed by quercetin. For the sugar moiety, the ^{1}H NMR spectrum displayed a doublets at δ 5.28 for the anomeric proton. Based on the *J* value of 6.0 Hz and comparison against literature data, the sugar was identified as α-L-arabinopyranose (Park *et al.*, 2012; Wang *et al.*, 2012). In addition, the position of the sugar was assigned on the C-3 of quercetin based on the observation of a cross-peak between $δ_H$ 5.28 (H-1") and $δ_C$ 103.1 (C-3) in the gHMBCAD spectrum (Figure S4). Based on these spectral evidence, compound **2** was identified as quercetin-3-*O*-α-L-

arabinopyranoside.

Compound **3** appeared as light yellow crystals. The ESI-MS spectrum of this compound acquired under negative ion mode displayed deprotonated molecular ion peak at m/z 447 and aglycone ion at m/z 300 (Figure S5). These data gave the molecular formula of $C_{21}H_{20}O_{11}$ for the whole compound and $C_{15}H_{10}O_7$ for the aglycone. These data suggested that compound **3** was a derivative of quercetin as well (Li *et al.*, 2016). Similar to compounds **1** and **2**, the ^{1}H and ^{13}C NMR spectra of compound **3** displayed the characteristic signals of quercetin in the aromatic region, further confirmed the identity of this compound as a quercetin derivative. The difference observed was due to the signals of the sugar unit attached to quercetin. The anomeric proton of the sugar unit was detected at δ 5.34 as a doublet with *J* value of 1.5 Hz. The small coupling constant suggested α-configuration of the sugar unit (Wang *et al.*, 2012; Yaya *et al.*, 2012). Furthermore, a doublet with *J* value of 6.5 Hz was detected at δ 0.93. This signal was integrated for three protons and found to be correlated to the sugar proton at $δ_H$ 3.43 (H-5") in the gCOSY spectrum (Figure S6A). Besides, a correlation between this signal and the sugar carbon at $δ_C$ 71.8 (C-5") was also observed in the gHMBCAD spectrum (Figure S6B). These indicated that this methyl group was part of the sugar. Based on these spectroscopic information and information available in the literature, this sugar was identified as α-L-rhamnose (Yaya *et al.*, 2012). The glycosylation of quercetin by this rhamnose unit was also revealed by the gHMBCAD spectrum to take place at the C-3 position, on the basis of the cross-peak observed between H-1" ($δ_H$ 5.28) and C-3 ($δ_C$ 103.1). Subsequently, compound **3** was characterised as quercetin-3-*O*-α-L-rhamnoside.

Compound **4** appeared as white needle crystals. The ^{1}H NMR spectrum of this compound demonstrated an aromatic signal which integrated for two protons. This suggested the presence of two aromatic proton which are symmetrical to one another (Mohd Nazrul *et al.*, 2011). Besides, the signal at δ 3.81 were integrated for three protons, indicating the presence of a methoxy group. The ^{13}C NMR spectrum also revealed the symmetrical structure of compound 4. Aromatic signals at δ 146.6 and δ 110.3 both arose from two carbons which were symmetrical to one another. The signal of a carbonyl carbon (δ 169.1) was also displayed, in addition to the methoxy group (δ 52.4). These spectral data was similar to those previously reported for methylgallate (Mohd Nazrul *et al.*, 2011; Choi *et al.*, 2014). This was further confirmed by the gHMBCAD spectrum,

Table 1. DPPH free radical scavenging and α-glucosidase inhibitory activities of isolated phenolic compounds.

Compound / Fraction	DPPH inhibition		α-Glucosidase inhibition	
	IC$_{50}$ (μM)	IC$_{50}$ (μg/mL)	IC$_{50}$ (μM)	IC$_{50}$ (μg/mL)
1	38.98 ± 0.94[A]	16.93 ± 0.41[A]	57.13 ± 2.19[A]	24.82 ± 0.95[A]
2	33.00 ± 0.97[B]	14.33 ± 0.42[B]	71.71 ± 4.44[B]	31.15 ± 1.93[B]
3	23.09 ± 0.70[C]	10.35 ± 0.27[C]	106.12 ± 1.71[C]	47.58 ± 0.76[C]
4	17.25 ± 0.23[D]	3.18 ± 0.04[a]	50.76 ± 0.92[A]	9.35 ± 0.17[a]
5	20.88 ± 0.60[E]	14.09 ± 0.43[b]	ND	ND
Quercetin	10.25 ± 0.42[F]	NR	7.35 ± 0.18[D]	NR
Ethyl acetate	NR	22.18 ± 1.33[D]	NR	2.27 ± 0.12[D]
Methanol	NR	10.04 ± 0.52[c]	NR	0.44 ± 0.06[b]

Values are means ± standard deviation of three replicates. For IC$_{50}$, the uppercase letters refer to the comparison of compounds **1**, **2** and **3** with ethyl acetate fraction; while the lowercase letters refer to the comparison of compounds **4** and **5** with methanol fraction. Mean with different letters are significantly different ($p < 0.05$). ND = not detected; NR = not related.

where a correlation between the methoxy proton and the carbonyl carbon was observed (Figure S7). Hence, based on these spectral evidence, compound **4** was unambiguously identified as methylgallate.

Compound **5** appeared as yellow amorphous powder. The ESI-MS spectrum of this compound in negative ion mode gave a deprotonated molecular ion peak at m/z 609, which corresponded to the molecular formula of $C_{27}H3_0O_{16}$ (Figure S8). The presence of aglycone ion at m/z 300 suggested compound **5** as a derivative of quercetin (Li *et al.*, 2016). The ^{1}H and ^{13}C NMR signals in the aromatic region were observed to be the same as compounds **1**, **2** and **3**. This further confirmed the presence of quercetin skeleton in compound **5**. However, there were more sugar signals detected in compound **5** as compared to compounds discussed earlier. The sugar signals detected corresponded to two sugar units. Anomeric protons were observed at δ 5.10 as doublet with *J* value of 7.5 Hz and at δ 4.51 as singlet. The signals of other sugar protons resonated between δ 3.35 and δ 3.79. At the high field δ 1.11, a doublet of methyl group was observed and found to be part of the sugar moiety based on its COSY and HMBC correlations with H-5‴ (Figure S9A) and C-5‴ (Figure S9B), respectively. These spectral information together with literature data (Sintayehu *et al.*, 2012; Lima *et al.*, 2014) revealed the presence of β-D-glucose and α-L-rhamnose.

Confirmation of the position where the sugar attached was obtained from the gHMBCAD spectrum of compound **5** (Figure S9B). A correlation was seen between the anomeric proton of β-D-glucose (δ 5.10) and the carbon signal at δ 134.2 (C-3), indicating that the β-D-glucose was attached to the quercetin at the C-3 position. On the other hand, a cross peak between anomeric proton of α-L-rhamnose and the carbon signal at δ 67.1 (C-6″ of the glucose) confirmed that the α-L-rhamnose was attached to the glucose at its

6″ position. This information confirmed the sugar moiety as a rutinoside and was in good agreement with available data (Lima *et al.*, 2014). Subsequently, compound **5** was characterised as quercetin-3-*O*-rutinoside (rutin). The individual chemical structures of compounds **1**–**5** are shown in Figure 1.
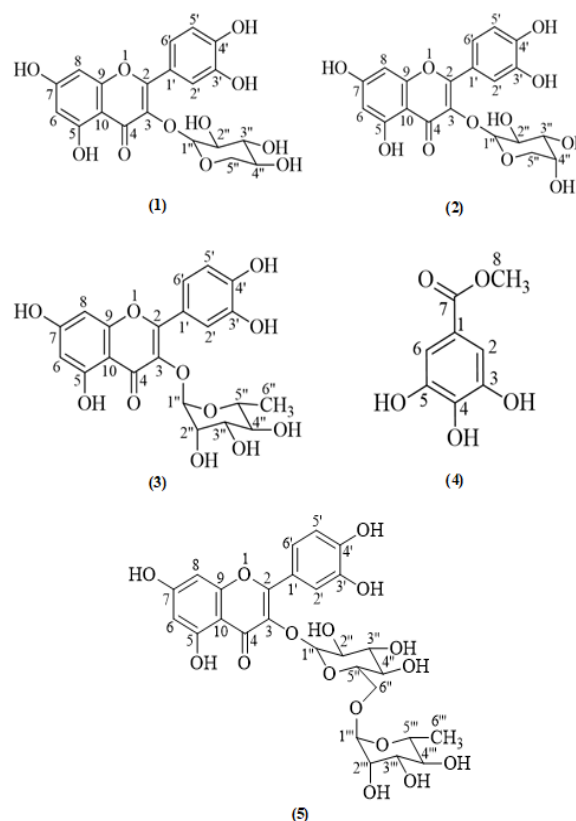

Figure 1. Chemical structures of isolated compounds **1**-**5**.

DPPH free radical scavenging and α-glucosidase inhibitory activities of isolated phenolic compounds

Five phenolic compounds were isolated from the EtOAc and MeOH fractions of *N. oleracea*. They were individually evaluated for their DPPH free radical scavenging and α-glucosidase inhibitory

activities to validate their contribution towards the tested bioactivities of *N. oleracea*, and the results are presented in Table 1. The results show that the isolated phenolic compounds were potential natural antioxidants. The antioxidant potential could be attributed to the presence of catechol moiety and extra hydroxyl groups in their structures. The scavenging activity was in the order of quercetin > **4** > **5** > **3** > **2** > **1**. Among the isolates, the gallic acid derivative, namely methylgallate (**4**) exhibited the most potent DPPH free radical scavenging activity, with the IC_{50} value of 17.25 μM, followed by the quercetin glycosides (compounds **1**, **2**, **3** and **5**). The quercetin glycosides inhibited the DPPH free radicals with IC_{50} ranged from 20.88 to 38.98 μM.

Comparing the quercetin glycosides with the quercetin standard, the results revealed that the glycosides had lower activity than the aglycone. Since all of these glycosides were 3-*O*-glycosides of quercetin, the influence of the antioxidant activity by the presence of free hydroxyl group at the C-3 position were demonstrated. Glycosylation of this hydroxyl group by different sugar moieties had negative effect on the antioxidant activity of the flavonoid. This effect was also reported by Kim *et al.* (2002). Among the four quercetin-3-*O*-glycosides, glycosylation by rutinose seemed to yield the least effect as rutin (**5**) possessed the highest antioxidant activity. This result was similar to those previously reported by Jo *et al.* (2009).

The α-glucosidase inhibitory activity of the isolated phenolic compounds was also investigated, and presented in Table 1. The inhibitory activity was in the order of quercetin > **4** > **1** > **2** > **3** > **5**. Similar to the antioxidant activity results, methylgallate (4) exhibited the most potent α-glucosidase inhibition, with the IC_{50} value of 50.76 μM, and followed by the quercetin glycosides (compounds **1**, **2** and **3**), with the IC_{50} values ranged from 57.13 to 106.12 μM. Unlike the antioxidant activity results, rutin (**5**) exhibited the lowest α-glucosidase inhibitory activity among the four quercetin-3-*O*-glycosides. Its IC_{50} value was not able to be determined at the highest concentration that could be prepared (0.16 mM).

Comparison between quercetin glycosides and the quercetin standard also revealed the weakening of α-glucosidase inhibitory activity by glycosylation at the C-3 position. This also showed the importance of the C-3-OH for the inhibition on α-glucosidase enzyme. This observation was in agreement with those previously reported by Jo *et al.* (2009). The reduced inhibitory activity on α-glucosidase after glycosylation may be due to the increased molecular size and polarity as well as the transfer to nonplanar

structure (Xiao *et al.*, 2013). These factors reduced the effectiveness of the glycosides to bind, thereby inhibiting the enzyme. These reasons could also explained why rutin (**5**) had the lowest α-glucosidase inhibitory activity when compared to other isolated quercetin glycosides.

Relating the tested bioactivities of the isolated phenolic compounds to those of the bioactive fraction in which they were isolated, the results of DPPH free radical scavenging activity showed that the IC_{50} values of the isolated phenolic compounds (in μg/mL) were lower than their respective fractions, except rutin (**5**). Although the IC_{50} of rutin was relatively higher, it was still close to that of MeOH fraction. These revealed the significance of the isolated phenolic compounds towards the antioxidant activity of the bioactive fractions. On the other hand, the results of the α-glucosidase inhibitory activity showed that the IC_{50} values of all the isolated phenolic compounds (in μg/mL) were much higher than those of their respective fractions. This indicated the lower efficiency of the individual isolated phenolic compounds for the inhibition on α-glucosidase enzyme, as compared to their respective fractions. The better α-glucosidase inhibitory activity of the fractions could be attributed to synergistic mechanism of these phenolic compounds, as well as with others that were isolated (Boath *et al.*, 2012).

Quantitative determination of isolated phenolic compounds by HPLC

The isolated phenolic compounds were also subjected to HPLC for their absolute quantification in the respective fractions in which they were isolated. The isolated phenolic compounds themselves were used as the standards for the quantitative analysis. In the developed HPLC method, 10 mmol methanesulfonic acid was used as the aqueous part of the mobile phase. The reason of using methanesulfonic acid is to buffer the mobile phase into acidic (pH 2). Phenolic compounds contain hydroxyl and carboxylic groups, which tend to deprotonate and cause rapid elution when the mobile phase is neutral or basic. A low pH of the mobile phase is therefore necessary to prevent deprotonation and to obtain chromatogram with better resolution and peak shape (Shou *et al.*, 2009). The 10 mmol methanesulfonic acid has a pH of 2, and hence it is a good choice for aqueous part of the HPLC mobile phase.

Figures 2A and 2B show the respective chromatograms of the EtOAc and MeOH fractions of *N. oleracea*; while the content of each of the isolated phenolic compounds is presented in Table 2. As shown in Figure 2A, the three isolated quercetin derivatives
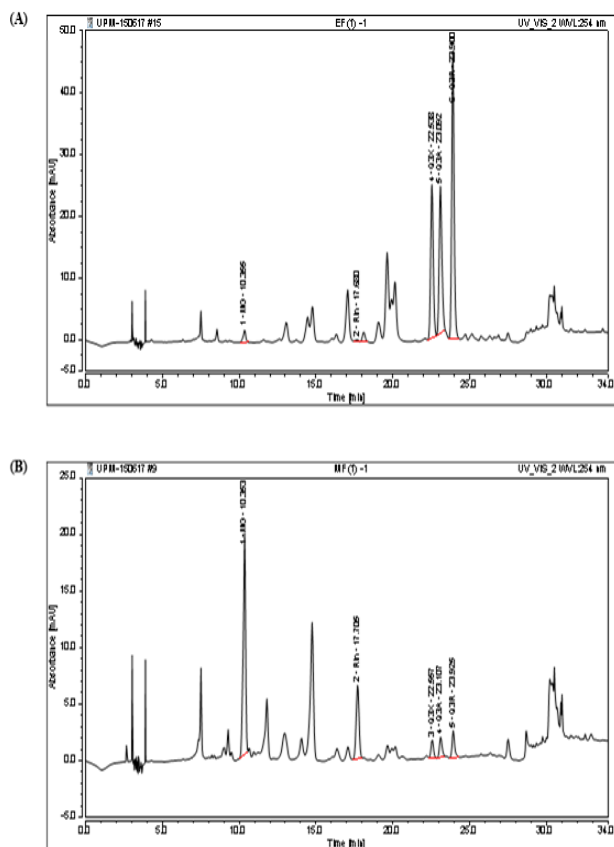
Figure 2. HPLC chromatograms of ethyl acetate (A) and methanol (B) fractions of *N. oleracea*.

Table 2. Quantitative analysis of isolated phenolic compounds by HPLC.

Compounds	Content in respective fraction (µg/mg)	
	Ethyl acetate fraction	Methanol fraction
1	131.10 ± 0.60[A]	ND
2	125.68 ± 0.73[B]	ND
3	157.55 ±0.41[C]	ND
4	ND	75.25 ±0.05[A]
5	ND	20.61 ± 0.02[B]
Total	414.33	95.86

Values are means ± standard deviation of three replicates. Mean with different letters are significantly different ($p < 0.05$). ND = not detected.

were the most prominent peaks in the EtOAc fraction, accounting for a total of 414.33 µg/mg. Quercetin-3-*O*-α-L-rhamnoside (**3**) was the most abundant among them, with the concentration of 157.55 µg/mg; followed by quercetin-3-*O*-β-D-xylopyranoside (**1**) and quercetin-3-*O*-α-L-arabinopyranoside (**2**) with the concentrations of 131.10 and 125.68 µg/mg, respectively. Meanwhile, methylgallate (**4**) was the most dominant peak in the MeOH fraction

(Figure 2B), and its concentration was much higher than that of rutin (**5**). Their content in the MeOH fraction was 75.25 and 20.61 µg/mg, respectively. The quantitative results obtained in the present work cannot be compared with the literatures as this is the first work on the quantification of the phenolic compounds present in *N. oleracea*. Nonetheless, this quantitative work provides information on the level of important components contained in the bioactive fractions of *N. oleracea*.

Conclusions

Five phenolic compounds, namely quercetin-3-*O*-β-D-xylopyranoside (**1**), quercetin-3-*O*-α-L-arabinopyranoside (**2**), quercetin-3-*O*-α-L-rhamnoside (**3**), methylgallate (**4**) and rutin (**5**) were isolated from *N. oleracea* for the first time. The structures of these compounds were elucidated using various spectroscopic techniques (MS, 1D and 2D NMR). Among these five compounds, methylgallate displayed the most prominent DPPH free radical scavenging and α-glucosidase inhibitory activities, with IC_{50} values of 17.25 and 50.76 µM, respectively. The significant contribution of the isolated phenolic compounds for the DPPH free radical scavenging of *N. oleracea* was also highlighted via the comparison of the bioactivities of the isolated compounds with their respective fractions. Meanwhile, synergistic effect was suggested for their contributions towards the α-glucosidase inhibitory activity. With the use of HPLC, the contents of the isolated phenolic compounds in their respective fraction were quantified, and the results revealed the high content of quercetin derivatives in EtOAc fraction (i.e., 131.10, 125.68 and 157.55 µg/mg for compounds **1**, **2** and **3**, respectively) and methylgallate (**4**) in MeOH fraction (75.25 µg/mg). The results presented in the present work provide information regarding the contribution of each isolated phenolic compounds towards the DPPH free radical scavenging and α-glucosidase inhibitory activities of *N. oleracea* as well as their contents in the plant.

Acknowledgement

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