NPC Natural Product Communications

2015
Vol. 10
No. 9
1561 - 1563

# Antioxidant and α-Glucosidase Inhibitory Constituents from *Hornstedtia* Species of Malaysia

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**Received: May 12th, 2015; Accepted: June 8th, 2015**

Seven compounds were isolated from the *n*-hexane and chloroform extracts of the flowers and leaves of four *Hornstedtia* species and their structures were identified using spectroscopic techniques as 3,7,4'-trimethylkaempferol (**1**), 3,7-dimethylkaempferol (**2**), 7,4'-dimethylkaempferol (**3**), 3,5-dimethylkaempferol (**4**), 3-methylkaempferol (**5**), stigmast-4-en-3-one (**6**), and 6-hydroxy-stigmast-4-en-3-one (**7**). Compounds **1** to **7** were isolated from these species for the first time. They were assayed for free radical scavenging and α-glucosidase inhibition activities. The DPPH assay showed that 3-methylkaempferol (**5**) was the most potent antioxidant agent with an $IC_{50}$ value 78.6 μM, followed by 7,4'-dimethylkaempferol (**3**) ($IC_{50}$ = 86.1 μM). For α-glucosidase inhibition activity, 3-methylkaempferol (**5**) exhibited significant inhibitory activity with an $IC_{50}$ value 21.0 μM. The present study revealed that *Hornstedtia* species have potential activities as antioxidant and α-glucosidase inhibitors.

**Keywords:** *Hornstedtia*, Zingiberaceae, Antioxidant, α-Glucosidase.

Free radicals are molecular species with a single unpaired electron. In spite of different types of free radicals, those of most concern in biological systems are the reactive oxygen species (ROS) [1]. The human body has an antioxidant defense system to cope with the activity of free radicals formed within the body. However, the imbalance between free radical production and the antioxidant defense arises when the free radicals are overproduced in the body [2]. This may result in the accumulation of oxidative stress and cause damage to macromolecules, such as lipids, proteins and nucleic acids. Oxidative damage to these molecular species leads to the development of various diseases, such as cardiovascular disease, cancer, cataract, diabetes mellitus and Parkinson's disease [3].

Diabetes mellitus has become a major health problem worldwide. The use of commercial α-glucosidase inhibitors (acarbose, miglitol and voglibose) is limited by their gastro-intestinal intolerability and high cost. Thus, the scientific community is searching for new natural compounds with anti diabetic properties to overcome any resistance developed by patients to the currently used drugs [4].

Numerous ginger species (Zingiberaceae) are widely used in South East Asia, not only because of their unique flavor but also because of their medicinal properties. *Hornstedtia* is a genus of Zingiberaceae which is placed in the tribe Alpinieae [5]. About 60 species of the genus have been recorded in tropical Asia [6]. The Javanese use the leaf of *Hornstedtia* species for flavoring, as well as to protect crops from insects by burning the leaves. In addition, in Kelantan, *H. macrocheilus* has been used as an external application to treat fever [7]. However, there is no previous phytochemical report on this genus from Malaysia, except for the extraction of the essential oils [8, 9]. Thus, this study was conducted to evaluate the antioxidant and α-glucosidase inhibitory activities of the compounds isolated from four Malaysian *Hornstedtia* species, *H. conica*, *H. havilandii*, *H. reticulata*, and *H. scyphifera*. Purification of the extracts of *Hornstedtia* species led to the isolation of five flavonoids,3,7,4'-trimethylkaempferol (**1**) [10], 3,7-dimethyl-kaempferol (**2**) [11], 7,4'-dimethylkaempferol (**3**) [12], 3,5-dimethylkaempferol (**4**) [13], and 3-methylkaempferol (**5**) [14] and two steroids, stigmast-4-en-3-one (**6**)and 6-hydroxy-stigmast-4-en-3-one (**7**) [15].

Radical scavenging action is an important attribute of antioxidants, which is measured by the DPPH radical scavenging assay. Compounds **1** to **5** were evaluated for their antioxidant activity and displayed various antioxidant activities according to their chemical structures (Table 1). Compounds **1** to **5** are kaempferol derivatives with different methoxylation patterns. Previous work reported strong antioxidant activity of kaempferol with free phenolic -OH groups (without any methoxyl substitution) with $IC_{50}$ 35.1 μM, suggesting the important role of the number of free phenolic groups in the antioxidant activity [16]. In this study, among five kaempferol derivatives, compound **5** with a methoxyl group at C-3 was the most active. The presence of free OH groups in compound **5** could be linked to higher antioxidant activity of the compound compared with other derivatives with more methoxylated groups. Methylation of free OH groups in flavonoids substantially reduces the antioxidant activity of the compounds [16]. Thus in agreement with this result, compound **3** with a 4'-OMe and 7-OMe displayed weak antioxidant activity compared with compound **5**. The free hydroxyl group at C-3 has been reported to be important for antioxidant activity, however compound **3** showed weak antioxidant activity [16]. This can be explained by the methylation of free -OH groups at C-4' and C-7 in compound **3**, which reduces the scavenging activity. Both compounds **2** and **4** showed antioxidant activity with $IC_{50}$ values of 93.2 and 90.7 μM, respectively. Compound **4** showed slightly higher antioxidant activity than compound **2** indicating that 5-OH seems to play a minor role in antioxidant activity. All compounds except **1** showed a change from

purple to yellow in DPPH solution. Compound **1** did not inhibit 50% of DPPH free radicals hence its $IC_{50}$ was higher than the highest concentration tested. The weak activity exhibited by this compound could be associated with its excessive methoxylation (3-OMe, 4'-OMe, and 7-OMe). Our results indicated that only compounds **2**, **3**, **4**, and **5** had significant activities, but these were still less than the positive controls, ascorbic acid (AA) and butylated hydroxytoluene (BHT).

**Table 1:** Percentage of antioxidant activity and the $IC_{50}$ values[a].

| Compound | Concentration (μM) | Percentage (%) | $IC_{50}$ (μM) |
|---|---|---|---|
| 1 | 100 | 22.5±0.1 | ND |
| 2 | 100 | 57.0±0.3 | 93.2±0.6 |
| 3 | 100 | 65.5±0.1 | 86.1±0.8 |
| 4 | 100 | 53.6±1.1 | 90.7±1.0 |
| 5 | 100 | 73.3±0.2 | 78.6±0.2 |
| BHT | 100 | 88.0±0.1 | 48.3±0.2 |
| AA | 100 | 94.9±0.3 | 4.3±0.1 |

[a]Data represent mean±standard deviation of four independent experiments; ND-not determined

Type 2 diabetes mellitus is an emerging health problem in which 90% of the cases are associated with individuals suffering with either obesity or who are overweight [17].α-Glucosidase inhibitors (e.g. acarbose, miglitol, and voglibose) can be used to retard the absorption of dietary carbohydrates [4]. These drugs act by reversible inhibition of α-glucosidase, an enzyme responsible for the breakdown of α-glycosidic bonds in complex carbohydrates to release absorbable monosaccharides [4]. Recent studies suggested that the increased free-radical production and reduced antioxidant defense may partially mediate the initiation and progression of diabetes-associated complications. Therefore, the presence of radical scavenging antioxidants can be useful in the prevention and/or reduction of the oxidative stress involved in this disease [18]. For this reason, this study was conducted to evaluate the α-glucosidase inhibitory activity of the isolated flavonoids which showed significant free radical scavenging activity.

Table 2 shows the inhibitory activity of flavonoids against α-glucosidase in the inhibition (%) at 100 μM. Compound **5** showed the most potent inhibitory activity with an $IC_{50}$ value of 21.3 μM compared with kaempferol with an $IC_{50}$ 12.0 μM [19]. This result indicated that the free phenolic OH at C-3 does not play a strong role in α-glucosidase inhibition activity.

**Table 2:** Percentage of α-glucosidase inhibition and the $IC_{50}$ values[a].

| Compound | Concentration (μM) | Percentage of Inhibition (%) | $IC_{50}$ (μM) |
|---|---|---|---|
| 1 | 100 | 57.0±1.1 | 80.5±1.6 |
| 2 | 100 | 85.1±0.1 | 43.3±0.3 |
| 3 | 100 | 51.2±1.5 | 78.74±0.2 |
| 4 | 100 | 89.6±0.3 | 35.4±0.1 |
| 5 | 100 | 95.1±0.20 | 21.0±0.1 |
| Acarbose | 100 | 98.1±0.02 | 3.4±0.1 |

[a]Data represent mean±standard deviation of four independent experiments; ND-not determined.

Compounds **2** and **4** displayed $IC_{50}$values of 43.1 and 35.1 μM, respectively. Compound **4** with a methoxyl group at C-5 displayed higher inhibitory activity than compound **2**, which suggests that methoxylation at C-5 does not affect the inhibitory activity. This finding was supported by the slight difference in inhibitory activity shown by fisetin and quercetin which showed that the absence of a 7-OH does not affect the inhibition activity [19]. Compounds **1** and **3** displayed weak α-glucosidase inhibition activity due to a minor role of free phenolic OH at C-3 and C-5 in the inhibition of α-glucosidase enzymes. A previous study reported insignificant correlation between the α-glucosidase inhibitory activity and total flavonoids. These results indicate that the inhibitory capacity against α-glucosidase enzymes depends on the content of phenolics rather than the flavonoid content of the extracts [20].

## Experimental

***General:*** $^{1}$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 spectrometerin $CDCl_3$, with TMS as an internal standard. The IR spectra were taken on a Perkin-Elmer series 1600 spectrophotometer as either a thin film (NaCl) window for liquid samples or a KBr pellet for solid samples. Vacuum liquid chromatography (VLC) was carried out using Merck silica gel 60 (230-400 mesh) and column chromatography (CC) using Merck silica gel 60 (70-230 mesh).

***Chemicals and reagents:*** Acarbose, α-glucosidase,and glycine were supplied by Merck (Darmstadt, Germany), while *p*-nitrophenyl-α-D-glucopyranose (PNPG) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich.

***Plant materials:*** Four species of *Hornstedtia* were studied, i.e *H. conica* Ridl., *H. havilandii* K. Schum., *H. reticulata* K. Schum. and *H. scyphifera* (J. Koenig) Steud. The leaves and flowers of *H. conica* were collected from Hutan Bukit Hantu, Kelantan in September 2011and *H. scyphifera* (leaves) from Bukit Fraser, Pahang in December 2013. These species were identified by Dr Shamsul Khamis from the Institute of Bioscience (IBS), Universiti Putra Malaysia (UPM). The plant specimens with voucher number, SK1976/11 (*H. conica*) and SK2324/14 (*H. scyphifera*) were deposited at the Herbarium of IBS, UPM. The leaves and flowers of *H. havilandii* and *H. reticulata* (leaves) were collected from Sarawak in March 2012. These species were collected by Mdm. Mohizar Mohamad and the voucher specimens (UiTMKS4007 for *H. havilandii* and UiTMKS4008 for *H. reticulata*) were deposited at the Natural Product Research & Development Centre (NPRDC), Universiti Teknologi Mara (UiTM), Sarawak.

***Extraction and isolation:*** Dried leaves of *H. conica*( 305.1 g), *H. havilandii* (361.0 g), *H. reticulata* (324.0 g), and *H. scyphifera* (795.9 g) and dried flowers of *H. conica* (321.8 g) and *H. havilandii* (202.9 g) were extracted using a Soxhlet extractor with *n*-hexane (5.0 L) and chloroform (5.0 L) for 18 h. The extract was filtered and concentrated using a rotary evaporator to give the crude extracts. Purification of these extracts was carried out with several chromatographic methods including vacuum liquid chromatography (VLC), column chromatography (CC), and Sephadex LH20 CC. Purification of the *n*-hexane extracts of the leaves of *H. havilandii* and *H. scyphifera* was carried out using CC and elution with *n*-hexane and $Et_2O$ (2:1) followed by recrystallization from *n*-hexane. This gave 3,7,4'-trimethylkaempferol **(1)** (25.9 mg, 0.24%, m.p. 130-133°C (lit. [10], 134-136°C) as a yellow solid. Subjecting the *n*-hexane extract of the leaves of *H. scyphifera* to silica gel CC (solvent system; Hex:$Et_2O$, 1:4) also yielded 3,7-dimethylkaempferol (**2**) (5.9 mg, 0.09%) as a yellow gum. Further purification of the *n*-hexane fraction of the leaves of *H. havilandii* by CC (solvent system; Hex:$Et_2O$, 3:2) afforded 7,4'-dimethylkaempferol **(3)** (17.2 mg, 0.16%, m.p. 180-181°C (lit. [21] 179-182°C) as yellow needles. 7,4'-Dimethylkaempferol **(3)** (117.5 mg, 3.39%) was also isolated as yellow needles from the *n*-hexane extract (117.5 mg, 3.40%, m.p. 180-181°C (lit. [21] 179-182°C) as yellow needles. The $CHCl_3$ fraction of the leaves of *H. reticulata* and *H. scyphifera* were subjected to silica gel CC and eluted with a stepwise gradient of *n*-hexane and diethyl ether (2:3) followed by Sephadex LH20 eluted with methanol afforded 3,5-dimethylkaempferol **(4)** (47.0 mg, 0.38%, m.p. 179-181°C (lit. [22] 180-181°C) as yellow crystals. Purification of the $CHCl_3$ fraction of *H. reticulata* using Sephadex LH20 (solvent; MeOH) gave 3-methylkaempferol (**5**) (34.8 mg, 0.29%, m.p. 269-275°C (lit. [23], 270-275°C) as a yellow solid. The *n*-hexane extracts of the flowers of *H. conica* and the leaves of *H. scyphifera* were purified using CC

eluted with *n*-hexane and diethyl ether (2:1) to yield stigmast-4-en-3-one (**6**) (5.0 mg, 0.11%) and 6-hydroxy-stigmast-4-en-3-one (**7**) (10.0mg, 0.21%), respectively.

***α-Glucosidase inhibition assay:*** The assay of α-glucosidase inhibition activity was performed as described by Sarmadi *et al.* [24]. The substrate was prepared by dissolving *p*-nitrophenyl-α-D-glucopyranoside (PNPG) in 50 mM phosphate buffer (pH 6.5), which is comparable to the condition of intestinal fluid. The test samples were prepared at 1000 ppm and 7 serial dilutions (1000, 500, 250, 125, 62.5, 31.3, and 15.6 µg/mL) were prepared. The test samples were mixed in a 96-well microplate and incubated at room temperature for 5 min. Fifty µL of PNPG was added to each well of sample, blank substrate, negative and positive controls and incubated for 15 min at room temperature. The reaction mixtures were stopped by using the stopping agent, 50 µL of 2M glycine (pH 10). The absorbance was measured at 405 nm and the α-glucosidase inhibition activity of the test sample was expressed as percentage of inhibition. The percentage of inhibition was calculated according to the following formula; % inhibition of sample = $[(A_n-A_s)/A_n]$ x 100%, where $A_n$ is the difference in absorbance of the negative control and all the blanks while $A_s$ the difference in absorbance of the sample and all the blanks.

***Free radical scavenging activity (DPPH):*** DPPH radical-scavenging capacity of flower extracts was evaluated according to the method of Li and Seeram [25] with slight modifications. The assay was performed in a 96-well microplate. Briefly, 50 µL aliquots of 8 serial dilutions (1000, 500, 250, 125, 62.5, 31.3, 15.63, and 7.81 µg/mL) of the test samples, in triplicate, were put in the wells. Then, 100 µL DPPH (80 mg/L) was added to each well. The mixture was then incubated in the dark for 30 min. Thereafter, the absorbance of the sample was measured using a micro-plate reader at 517 nm. The radical scavenging activity was examined and compared with ascorbic acid and butylated hydroxytoluene (BHT) as the references. The percentage of DPPH discoloration of the test sample was calculated according to the following equation; I% = $[(A_o-A_s)/A_o]$ x 100 where $A_o$ is the absorbance of the reagent blank and $A_s$ is the absorbance of the test samples. All tests were performed in triplicate. The results were expressed in $IC_{50}$ values.

**Acknowledgements** - The authors wish to thank Universiti Teknologi Malaysia for research facilities and Ministry of Education (MOE) for financial support under research grant QJ130000.2526.04H48 and MyBrain15 for the scholarship to Siti Ernieyanti Hashim.

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