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Identification of α -glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less, a traditional Indonesian herb: Promotion of natural product use

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Identification of α -glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less, a traditional Indonesian herb: Promotion of natural product use

A promising approach for treating diabetes mellitus (DM) is to decrease postprandial hyperglycemia by suppressing carbohydrate digestion using α -glucosidase inhibitors. *Pluchea indica* leaf extracts have inhibitory activity against intestinal maltase. Enzyme assay-guided fractionation by chromatography yielded five active caffeoylquinic acid derivatives (1-5). Their structures were elucidated by mass spectrometry and NMR analysis and completed by comparison with reference data. 3,5-di-*O*-caffeoylquinic acid (1), 4,5-di-*O*-caffeoylquinic acid methyl ester (2), 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (3), 3,4,5-tri-*O*-caffeoylquinic acid (4), and 1,3,4,5-tetra-*O*-caffeoylquinic acid (5) were isolated. Comparison of the activities of each isolate suggested that both methyl esterification of quinic acid and the number of caffeate groups in the molecule were important to the inhibitory activity. This study provides basic information for further examination the suitability of *P. indica* as a functional food and medicinal supplement for the treatment and prevention of diabetes.

Keywords: Indonesian herb, intestinal maltase inhibitor, diabetes mellitus, *Pluchea indica*, caffeoylquinic acid derivatives.

1. Introduction

An effective tool in the management of diabetes mellitus (DM), and particularly that of non-insulin-dependent diabetes mellitus (NIDDM), is to decrease postprandial hyperglycemia by inhibiting α -glucosidase in the digestive system. Many efforts have been made to identify α -glucosidase inhibitors from indigenous plants to develop physiologically functional foods and to discover compounds for use against diabetes (Kumar et al. 2011). Some indigenous plants from Indonesia have α -glucosidase inhibitory activity, e.g., *Macaranga tanarius* leaves (Gunawan-Puteri & Kawabata 2010) and *Eleutherine americana* bulbs (Ieyama et al. 2011). We screened twenty-five Indonesian medicinal herbs for their α -glucosidase inhibitory activity, particularly intestinal maltase inhibitory activity. *Pluchea indica* (L.) Less (Asteraceae) was found to be a promising source of α -glucosidase inhibitors.

P. indica is widely distributed in southeast Asia (Raharjo & Horsten 2001). Extracts of *P. indica* parts have anti-oxidant, anti-ulcer, anti-nociceptive, anti-diuretic, and anti-inflammatory properties (Sen & Chaudhuri 1991; Choi & Hwang 2005; Biswas et al. 2007; Buapool et al. 2013). In Thailand, *P. indica* leaves are used as tea because

they are believed to have an indigenous remedy due to their anti-diabetic properties. It has been reported for its anti-diuretic and anti-diabetic pharmacological effects in streptozocin-induced rats (Pramanik et al. 2006). Prior chemical investigations of *P. indica* have led to the isolation of several terpenes, lignin glycosides and terpenic glycosides from the aerial part (Uchiyama et al. 1991; Raharjo & Horsten 2001). The methanolic extract of the *P. indica* roots led to the isolation of an alkynylthiophene derivative 2-(prop-1-ynyl)-5(5,6-dihydroxyhexa-1, 3-diy-nyl)-thiophene for its anti-amoebic activity (Biswas et al. 2007). 3,4,5-tri-*O*-caffeoylquinic acid and 1,3,4,5-tetra-*O*-caffeoylquinic acid, herein referred to as compounds **4** and **5** respectively, were recently isolated as constituents for this plant and have been reported to show collagenase inhibitory activity (Ohtsuki et al. 2008). However, *P. indica* is not used as an anti-diabetic agent in Indonesia. The compounds in *P. indica* responsible for α -glucosidase inhibition are unknown. Therefore, this study was performed to discover the compounds responsible for the inhibition of α -glucosidase, specifically of intestinal maltase, using a bio-assay-guided approach.

2. Results and Discussion

Compounds were extracted from dried leaves of *P. indica* using 50% methanol. The methanol extract was partitioned between ethyl acetate (EtOAc) and water. The EtOAc fraction demonstrated a higher inhibitory activity against maltase than the water fraction. Further fractionation was carried out by chromatography and by guidance of the rat intestinal maltase inhibitory activity.

The EtOAc fraction was subjected to silica gel column chromatography using chloroform-methanol gradient and the active fraction was further fractionated using ODS column chromatography. The active ODS fractions were further purified using preparative HPLC resulting in the isolation of five caffeoylquinic acid derivatives: 3,5-di-*O*-caffeoylquinic acid (**1**), 4,5-di-*O*-caffeoylquinic acid methyl ester (**2**), 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**), 3,4,5-tri-*O*-caffeoylquinic acid (**4**), and 1,3,4,5-tetra-*O*-caffeoylquinic acid (**5**). The structure of all isolates (**1–5**) was determined using mass spectrometry and ¹H-NMR spectra. The NMR pattern of all compounds showed characteristic signals of caffeoylquinic acids and their structures were finally confirmed using published spectra: **1** (Basnet et al. 1996; Gao et al. 2008), **2** (Gao et al. 2008), **3** (Mertfort 1992), **4** (Islam et al. 2002) and **5** (Scholz et al. 1994).

This is the first report for the identification of compounds **1–3** from *P. indica*, whereas compounds **4** and **5** were recently isolated as constituents for this plant and have been reported to show collagenase inhibitory activity (Ohtsuki et al. 2008). Interestingly, the 3,5-isomer of dicaffeoylquinic acid was isolated as a free form (**1**), whereas the 4,5-isomer was isolated only as a methyl ester (**2**). This indicates that methyl esterification selectively occurs in plant tissues. Compounds **1** and **4** have been previously reported as maltase inhibitors (Matsui et al. 2004). However, compounds **2**, **3**, and **5** have not been evaluated for their intestinal maltase inhibitory activity.

The isolated caffeoylquinic acid derivatives were compared for their rat intestinal maltase inhibitory activity (Table 1). Half maximal inhibitory concentration (IC₅₀) values were used as measures of the effectiveness of each compound to inhibit maltase function. On the basis of IC₅₀ values, compound **3** had the highest inhibition among the caffeoylquinic acid derivatives isolated from *P. indica* leaves, followed by **4**, **5**, **2**, and **1** respectively. This suggests that, when comparing the inhibitory activity of compounds **1**, **4**, and **5**, increasing numbers of caffeoyl groups attached to quinic acid moiety enhanced maltase inhibitory activity. This is consistent with a previous study in which the caffeoyl group played an important role in intestinal α -glucosidase inhibitory activity (Matsui et al. 2004). Among the compounds with the same number of caffeoyl groups, compounds **2** and **4** had five-fold higher inhibitory activities than compounds **1** and **3**, respectively. This suggests that methyl esterification of the carboxylic group in quinic acid has an additional effect of enhancing maltase inhibitory activity. This is the first report for the importance of the methyl esterification of caffeoylquinic acids with respect for their α -glucosidase inhibitory activity, although the mechanism still remains undetermined.

In the present study, we showed that increases in maltase inhibitory activity were due both to the number of caffeoyl groups attached to the quinic moiety and to the presence of methyl esterification on the carboxyl group. Considering the yields and IC₅₀ values of the caffeoylquinic acid derivatives isolated, the inhibitory activity of *P. indica* leaves is likely to be due to compounds **3**, **4**, and **5**, although compounds **1** and **2** may also contribute to the activity.

The caffeoylquinic acid derivatives (**1–5**) isolated from *P. indica* were intestinal maltase inhibitors. By comparing the inhibitory activities of the isolates, we determined that methyl esterification of the quinic acid moiety contributes to the inhibitory activity of the compound. We also determined that the number of caffeoyl groups in the

molecule contributes to the inhibitory activity. Caffeoylquinic acid derivatives from this plant may be important medicinal substances, which may delay postprandial hyperglycemia. *Pluchea indica* is an indigenous plant that may potentially be used as a functional food or as a medicinal supplement for diabetes mellitus treatment and prevention.

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SUPPLEMENTARY MATERIAL

Identification of α -glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less, a traditional Indonesian herb: Promotion of natural product use

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Abstract

A promising approach for treating diabetes mellitus (DM) is to decrease postprandial hyperglycemia by suppressing carbohydrate digestion using α -glucosidase inhibitors. *Pluchea indica* leaf extracts have inhibitory activity against intestinal maltase. Enzyme assay-guided fractionation by chromatography yielded five active caffeoylquinic acid derivatives (**1-5**). Their structures were elucidated by mass spectrometry and NMR analysis and completed by comparison with reference data. 3,5-di-*O*-caffeoylquinic acid (**1**), 4,5-di-*O*-caffeoylquinic acid methyl ester (**2**), 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**), 3,4,5-tri-*O*-caffeoylquinic acid (**4**), and 1,3,4,5-tetra-*O*-caffeoylquinic acid (**5**) were isolated. Comparison of the activities of each isolate suggested that both methyl esterification of quinic acid and the number of caffeate groups in the molecule were important to the inhibitory activity. This study provides basic information for further examination the suitability of *P. indica* as a functional food and medicinal supplement for the treatment and prevention of diabetes.

Keywords: Indonesian herb, intestinal maltase inhibitor, diabetes mellitus, *Pluchea indica*, caffeoylquinic acid derivatives.

Experimental Procedures

1. Materials

Pluchea indica leaves were purchased from Merapi Farma Traditional Herbs Distributor, Yogyakarta, Indonesia, in January 2007. A voucher specimen (batch number B0107.002) was deposited with the Merapi Farma Herbal Traditional Herbs Distributor. Rat intestinal acetone powder was supplied by Sigma Aldrich Japan Co. (Tokyo, Japan). Cosmosil 75C₁₈-OPN was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All chemicals used were of analytical grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan), unless otherwise stated.

2. Instrumental analysis

¹H-NMR spectra were recorded using Bruker AMX500 spectrometer (500 MHz). Chemical shifts were calculated from the residual solvent signal (methanol-*d*₄, δ_{H} 3.30). Mass spectrum was obtained using Thermo Scientific Exactive spectrometer or Waters LCT Premier XE. High-pressure liquid chromatography (HPLC) was performed with a JASCO 802-SC system (JASCO Co., Tokyo, Japan).

3. Rat intestinal maltase inhibitory activity assay

Rat intestinal maltase inhibitory activity was determined using the method of Jong-Anurakkun et al. (2007), with minor modifications. Rat intestinal acetone powder was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM ethylenediaminetetraacetic acid (EDTA), homogenized, and centrifuged at $10,600 \times g$ for 30 min at 4°C. The supernatant was collected and used as the crude enzyme solution. The maltose-hydrolyzing activity of the crude enzyme solution (2.33 U/mg protein) was designated as maltase activity.

Inhibition of maltose hydrolysis was measured using the following procedure: Test tubes containing 350 μl maltose solution (3.5 mM) in potassium phosphate buffer (0.1 M, pH 6.3) were preincubated at 37°C for 5 min. Plant extracts were dissolved in 50% dimethyl sulfoxide (DMSO, 100 μl) and added to preincubated tubes. Control tubes contained 50% DMSO alone (100 μl). The reaction was started by the addition of crude rat intestinal glucosidase solution (50 μl). Test tubes containing 400 μl potassium phosphate buffer (0.1 M, pH 6.3) served as blanks. After incubation for 15 min at 37°C,

the reaction was stopped by adding 0.75 ml of 2 M Tris HCl buffer (pH 7.0). The reaction mixture was passed through a short ODS column (Cosmosil 75C₁₈-OPN, ϕ 5 × 55 mm) to remove phenolic compounds that might interfere with the glucose quantification. The amount of liberated glucose was measured by the glucose oxidase method using a commercial test kit (Glucose CII-test Wako, Wako Pure Chem. Co., Osaka, Japan) in 96-well microplates at 37°C for 30 min. The optical density (OD) of the wells was measured at 490 nm. Inhibitory activity was calculated using the following equation (1):

$$\text{Inhibitory activity (\%)} = \frac{[(\text{OD}_{\text{control}} - \text{OD}_{\text{control blank}}) - (\text{OD}_{\text{sample}} - \text{OD}_{\text{sample blank}})]}{(\text{OD}_{\text{control}} - \text{OD}_{\text{control blank}})} \times 100 \quad (1)$$

All experiments were performed in duplicate and the results were presented as half of the maximal inhibitory concentration value (IC₅₀ value). Acarbose, which had 50% inhibitory activity against maltase at 0.5 μ M concentration, was used as the positive control.

4. Isolation of intestinal maltase inhibiting principles from *P. indica*

Compounds were extracted from dried leaves of *P. indica* (94 g) with 50% aqueous methanol for 24 h at room temperature and filtered. Evaporation of the solvent under reduced pressure yielded a 50% methanol extract (16.18 g). This extract was partitioned between ethyl acetate (EtOAc) and water. Each layer was evaporated at reduced pressure to produce a EtOAc-soluble fraction (2.98 g) and water-soluble fraction (13.2 g).

The EtOAc-soluble fraction was applied to a silica gel (Silica Gel 60 N, Kanto Chemical Co. Inc. Tokyo, Japan) column (ϕ 3 × 42 cm) with a chloroform-methanol gradient and yielded 8 fractions. The eluent (volume and dry weight of eluate) of each fraction was as follows: fraction 1, chloroform (300 ml, 19 mg); fraction 2, 10% methanol in chloroform (150 ml, 27 mg); fraction 3, 10% methanol in chloroform (150 ml, 767 mg); fraction 4, 15% methanol in chloroform (150 ml, 196 mg); fraction 5, 15% methanol in chloroform (150 ml, 128 mg); fraction 6, 20% methanol in chloroform (150 ml, 189 mg); fraction 7, 20% methanol in chloroform (150 ml, 125 mg); and fraction 8,

methanol (500 ml, 1.51 g). Fraction 8 was active and further fractionated using ODS column chromatography (Cosmosil 75C₁₈-OPN, ϕ 3 \times 30 cm) with a water-methanol gradient and yielded 9 fractions. The eluent of each fraction was as follows: fraction 8-1, 25% methanol in water (200 ml, 110 mg); fraction 8-2, 25% methanol in water (200 ml, 221 mg); fraction 8-3, 25% methanol in water (200 ml, 94 mg); fraction 8-4, 50% methanol in water (200 ml, 342 mg); fraction 8-5, 50% methanol in water (200 ml, 189 mg); fraction 8-6, 50% methanol in water (200 ml, 57 mg); fraction 8-7, methanol (200 ml, 72 mg); fraction 8-8 methanol (200 ml, 63 mg); and fraction 8-9, methanol (200 ml, 43 mg). Fractions 8-4 and 8-5 were active and further purified by reversed-phase preparative HPLC. Fraction 8-4 was subjected to preparative HPLC (column: Inertsil PREP-ODS, 20.0 \times 250 mm, GL Science Inc.; mobile phase, water-methanol 60:40 to 40:60 (0-50 min) containing 0.1% of formic acid; flow rate 5 ml/min; detection: UV 254 nm) and yielded 10 mg of 3,5-di-*O*-caffeoylquinic acid (**1**, t_R 32 min), 75 mg of 4,5-di-*O*-caffeoylquinic acid methyl ester (**2**, t_R 35 min), 42 mg of 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**, t_R 47 min), and 41 mg of 1,3,4,5-tetra-*O*-caffeoylquinic acid (**4**, t_R 50 min). Fraction 8-5 was subjected to preparative HPLC in a manner similar to the purification of fraction 8-4, except water:methanol (60:40) containing 0.1% of formic acid was used as a mobile phase. Fraction 8-5 yielded 19 mg of 3,4,5-tri-*O*-caffeoylquinic acid (**5**, t_R 17.5 min). The purity of the material was confirmed by HPLC and NMR analyses.

3,5-di-*O*-caffeoylquinic acid (1), off-white powder, ESI-TOF-MS (negative) m/z 515.13 [M-H]⁻; ¹H NMR δ (methanol-*d*₄): 2.12-2.33 (4H, m, 2 and 6-H), 3.96 (1H, dd, $J = 7.3$ Hz, 3.5 Hz, 4-H), 5.41 (1H, m, 5-H), 5.42 (1H, m, 3-H), 6.26 and 6.34 (each 1H, d, $J = 16.0$ Hz, 2 \times 8'-H), 6.77 and 6.88 (each 1H, d, $J = 8.0$ Hz, 2 \times 5'-H), 6.95 and 6.96 (each 1H, brd, $J = 8.1$ Hz, 2 \times 6'-H), 7.05 and 7.06 (each 1H, d, $J = 2.2$ Hz, 2 \times 2'-H), 7.57 and 7.61 (each 1H, d, $J = 16.0$ Hz, 2 \times 7'-H).

4,5-di-*O*-caffeoylquinic acid methyl ester (2), light yellow powder, ESI-MS (negative): m/z 529.14 [M-H]⁻; ¹H NMR δ (methanol-*d*₄): 2.07 and 2.32 (2H, dd, $J = 16.0$ Hz, 5.0 Hz, 2-H), 2.28 (2H, m, 6-H), 3.71 (3H, s, OCH₃), 4.40 (1H, m, 3-H), 5.12 (1H, dd, $J = 8.2$ Hz, 3.2 Hz, 4-H), 5.54 (1H, ddd, $J = 13.0$ Hz, 8.2, 4.0 Hz, 5-H), 6.16 and 6.28 (each 1H, dd, $J = 16.0$ Hz, 2 \times 8'-H), 6.75 (2H, d, $J = 8.2$ Hz, 2 \times 5'-H), 6.90 and 6.91 (each 1H, dd, $J = 8.2$ Hz, 2.0 Hz, 2 \times 6'-H), 7.00 and 7.02 (each 1H, $J = 2.0$ Hz, 2 \times 2'-H), 7.50 and 7.60 (each 1H, d, $J = 16.0$ Hz, 2 \times 7'-H).

3,4,5-tri-*O*-caffeoylquinic acid methyl ester (3), light yellow powder, ESI-MS (negative): m/z 691.17 [M-H]⁻; ¹H NMR δ (methanol-*d*₄): 2.13 and 2.84 (each 1H, m, 2 \times 6-H), 2.39 and 2.45 (each 1H, dd, $J = 13.5$ Hz, 3.8 Hz, 2-H), 3.73 (3H, s, OMe), 5.33 (1H, dd, $J = 7.6$ Hz, 3.0 Hz, 4-H), 5.57 (1H, m, 3-H), 5.64 (1H, m, 5-H), 6.19, 6.23, and 6.29 (each 1H, d, $J = 16.0$ Hz, 3 \times 8'-H), 6.71, 6.75, and 6.75 (each 1H, dd, $J = 8.2$ Hz, 3 \times 5'-H), 6.86, 6.91, and 6.91 (each 1H, dd, $J = 8.2$ Hz, 1.9 Hz, 3 \times 6'-H), 7.00, 7.02, and 7.02 (each 1 H, d, $J = 1.9$ Hz, 3 \times 2'-H), 7.52, 7.52, and 7.58 (each 1H, d, $J = 16.0$ Hz, 3 \times 7'-H).

3,4,5-tri-*O*-caffeoylquinic acid (4), yellowish white powder, ESI-MS (negative): m/z 677.12 [M-H]⁻; ¹H NMR δ (methanol-*d*₄): 2.06-2.47 (4H, m, 2,6-H), 5.31 (1H, dd, $J = 8.4$ Hz, 3.5 Hz, 4-H), 5.67 (2H, m, 3,5-H), 6.22, 6.23, and 6.29 (each 1H, d, $J = 16$ Hz, 3 \times 8'-H), 6.68-6.85 (4H, m, 3 \times 5'-H, 6'-H), 6.92 (2H, m, 2 \times 6'-H), 6.98, 7.02, and 7.06 (each 1 H, br s, 3 \times 2'-H), 7.51, 7.53, and 7.60 (each 1H, d, $J = 16$ Hz, 3 \times 7'-H).

1,3,4,5-tetra-*O*-caffeoylquinic acid (5), yellowish-white amorphous powder, ESI-MS (negative): m/z 839.18 [M-H]⁻; ¹H NMR δ (methanol-*d*₄): 2.17 and 2.73 (2H, br d, $J = 13.0$ Hz, 6-H), 2.60 (1H, br dd, $J = 16.0$ Hz, 2.0 Hz, 2ax-H), 2.94 (1H, m, 2eq-H), 5.37 (1H, dd, $J = 10.3$ Hz, 3.6 Hz, 4-H), 5.73 (1H, m, 3-H), 5.86 (1H, ddd, $J = 11.5$ Hz, 10.3 Hz, 4.5 Hz, 5-H), 6.15, 6.24, 6.37, and 6.37 (each 1H, d, $J = 15.7$ Hz, 4 \times 8'-H), 6.05, 6.66, 6.73, and 6.73 (each 1H, d, $J = 8.2$ Hz, 4 \times 5'-H), 6.78, 6.78, 6.92, 6.92 (each 1H, dd, $J = 8.2$ Hz, 2.2 Hz, 4 \times 6'-H), 6.67, 6.94, 7.01, 7.07 (each 1H, d, $J = 2.2$ Hz, 4 \times 2'-H), 7.47, 7.49, 7.57, and 7.65 (each 1H, d, $J = 15.7$ Hz, 4 \times 7'-H).

Table 1. Yields and IC₅₀ values of intestinal maltase inhibitory activity for compounds isolated from *P. indica*.

No.	Compounds	Yield (%)	IC ₅₀ (μM)
1	3,5-di- <i>O</i> -caffeoylquinic acid	0.01	1166
2	4,5-di- <i>O</i> -caffeoylquinic acid methyl ester	0.08	208
3	3,4,5-tri- <i>O</i> -caffeoylquinic acid methyl ester	0.04	2
4	3,4,5-tri- <i>O</i> -caffeoylquinic acid	0.02	13
5	1,3,4,5-tetra- <i>O</i> -caffeoylquinic acid	0.04	11