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ORIGINAL PAPER

Pyranocarbazole alkaloids, isolated from *Clausena cambodiana* leaves, as a pancreatic cholesterol esterase inhibitor, and their HPLC–DAD quantitative determination method

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Abstract
A bioassay-guided isolation method was performed with pancreatic cholesterol esterase as a target enzyme. Four pyranocarbazole alkaloids, 8-hydroxymahanimbine (**1**), mahanine (**2**), mahanimbicine (**3**) and mahanimbine (**4**) were isolated from *Clausena cambodiana* Guill leaves for the first time. Furthermore, compound **1** has never before been isolated as a natural product. The chemical structures of the isolated compounds were elucidated by comprehensive spectroscopic analysis, including 1D and 2D-NMR, and comparison with published data. Compounds **1** and **2** exhibited potent inhibitory activity against pancreatic cholesterol esterase, with IC_{50} values of 48.56 and 34.56 µg/mL, respectively. The HPLC–DAD method for the quantification of four pyranocarbazole alkaloids from *C. cambodiana* leaves was also validated. The method was simple, accurate, rapid and reliable, and can be used for the quality control of *C. cambodiana* leaves. Our findings indicate that *C. cambodiana* leaves could be used as a functional food or pharmaceutical ingredient for the prevention and treatment of hypercholesterolemia.

Keywords Pyranocarbazole alkaloid · Pancreatic cholesterol esterase · *Clausena cambodiana* · Bioassay-guided isolation · HPLC–DAD

Introduction

Hypercholesterolemia is a disorder characterized by high levels of blood cholesterol (Ghule et al. 2009; Cho et al. 2006; Sergent et al. 2012). Cholesterol is produced primarily in the liver and then carried to the cells throughout the body by low-density lipoprotein (LDL) (Thilakarathna et al. 2013). Lipoproteins, particles formed in the liver, play a role in the transportation of cholesterol and other fats through the bloodstream. All lipoproteins carry cholesterol, but elevated levels of lipoproteins other than high-density lipoprotein (HDL), particularly LDL cholesterol, are associated with an increased risk of atherosclerosis and coronary heart disease (Handa et al. 2013; Al-Shaer et al. 2004; Ngamukote et al. 2011).

Pancreatic cholesterol esterase is an important enzyme that plays a significant role in the absorption of dietary cholesterol. The inhibition of pancreatic cholesterol esterase has attracted much attention in recent decades as a potential means of treating hypercholesterolemia and atherosclerosis by limiting the bioavailability of dietary cholesterol (Wei et al. 2014). Simvastatin has already been proved to be useful as a cholesterol-lowering drug that blocks the enzyme HMG-CoA reductase, which is required for the biosynthesis of cholesterol (Endo 2008). It has also been reported to inhibit cholesterol esterase in vitro and in vivo (Chiou et al. 2006). However, this synthetic drug can cause a range of adverse effects, including muscle complications and

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an increased risk of diabetes (Endo 2008). Therefore, the search for a natural, safe and highly effective cholesterol esterase inhibitor produced from botanical materials may be beneficial.

The biodiversity of plants can provide an endless resource for possible drugs and the long history of folk medicines demonstrates the potential use of plants as sources of cholesterol-lowering agents (Lemhadri et al. 2006). In Southern Thailand, people typically eat an abundance of raw fresh vegetables (Taungbudhitham 1995), and some edible plants specifically grown in the region, such as Phlu (*Piper betle* Linn), Sadao Chang (*Azadirachta excelsa* (Jack) Jacobs), and Samui Hom (*Clausena cambodiana* Guill), are used as traditional medicines. People in Southern Thailand believe that compounds from these plants have anti-diabetic and cholesterol-lowering properties. However, there has been little scientific evidence to support these claims.

Our preliminary screenings for anti-pancreatic cholesterol esterase activity from some edible plants in Southern Thailand revealed that eight ethanolic plant extracts inhibited pancreatic cholesterol esterase. However, an ethanol extract of *Clausena cambodiana* leaves showed the highest inhibitory activity against pancreatic cholesterol esterase (Sakunpak et al. 2016). A member of the Rutaceae family, *C. cambodiana* is distributed throughout Southern Thailand. The young leaves and shoots are edible and are traditionally used in the treatment of fever and dizziness, while a poultice of the boiled roots can be used to relieve skin irritation. The roots and shoots of *C. cambodiana* have been recognized as a rich source of various phytochemicals, such as clausarin, clausenidin, dentatin, nordentatin, xanthoxyletin, dihydropyranocoumarin, quercetin, sinensetin and β-sitosterol (Tangyuenyongwatthana and Thanayavuthi 1992). Some of these compounds demonstrate notable biological activities, including antimalarial, anti-tuberculosis, cytotoxic, and anti-diabetic properties (Maneerat et al. 2013; Damsud et al. 2017). As the active components of *C. cambodiana* leaves putatively responsible for retarding pancreatic cholesterol esterase have not been investigated, in this study, we isolated the relevant active compounds of the leaves and assessed their relative effectiveness against pancreatic cholesterol esterase by determinations of their IC_{50}. The biomarker compounds were then quantified using the HPLC–DAD method.

Materials and methods

Chemicals and reagents

Methanol (HPLC grade) was purchased from Labscan Asia (Bangkok, Thailand). Ultrapure water was obtained from the Puris, Expe-UP water system (Korea; resistivity 18.2 MΩ cm). Ethanol, *n*-hexane, dichloromethane and ethyl acetate were obtained from Carlo Erba (Milano, Italy). Pancreatic cholesterol elastase (Lot.No. 26745), *p*-nitrophenyl butyrate (*p*-NPB), orlistat, simvastatin, ferrous sulfate, taurocholic acid sodium salt, sodium acetate, sodium chloride, sodium citrate, dibasic sodium phosphate and monobasic sodium phosphate were purchased from Sigma-Aldrich (USA).

General experimental procedures

One- and two-dimensional nuclear magnetic resonance (NMR) spectra were recorded on a BRUKER Fourier 300 spectrometer (Bruker Corporation, Bremen, Germany). Tetramethylsilane (TMS) was used as an internal standard. Mass spectra were acquired by the use of a MALDI-TOF mass spectrometer (JEOL, Tokyo, Japan). Preparative HPLC was performed using a Young Lin apparatus equipped with a binary pump (YL9111S) and a PDA detector (YL9160S). The quantitative analysis of biomarker compounds was conducted using a PFP column (Agilent 1200 series, Agilent Poroshell 120 Pentafluorophenyl column, 4.6 × 50 mm, 2.7 µm). Infrared spectra were recorded with a Spectrum One FT-IR spectrometer (Perkin Elmer, Shelton, USA), and the wavelengths (λ) are measured in cm^{-1}.

Plant materials

Clausena cambodiana leaves were collected from Phatalung, Trang, Yala and Songkhla Provinces, Thailand, in June 2019. A voucher specimen (SKP 166 03 03 01) was deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The plant was dried at 50 °C in a hot air oven for 24 h before being reduced to a coarse powder using a grinder.

Bioassay-guided isolation

Dried plant powder (1500 g) was extracted twice with ethanol (2 × 2 L) while sonicated for 1 h. The extracts were combined and concentrated under reduced pressure to obtain a dark green extract (350 g), which was fractionated using silica gel column vacuum chromatography (15 × 20 cm). *n*-Hexane, dichloromethane, ethyl acetate and methanol were used as the mobile phase, using a step gradient elution. The four resulting fractions were hexane (28 g), dichloromethane (54 g), ethyl acetate (83 g) and methanol (152 g). After that, all fractions were subjected to tests for pancreatic cholesterol esterase inhibitory activity. The ethyl acetate fraction, which showed the highest percentage of inhibition of the enzyme, was further purified by a Diaion HP-20 column (10 × 80 cm) eluted with a mixture of methanol and water using a step gradient elution (10–100% methanol), to give eleven pooled fractions (fractions 1–11). The fractions were then subjected

to an enzyme inhibition assay. Compounds **1** (38.2 mg), **2** (45.4 mg), **3** (15.1 mg) and **4** (56.5 mg) were obtained from fraction five after being purified using preparative RP-C18 HPLC column (ACE C18-300 column) and a mixture of water and methanol (10:90 v/v) as eluent, with a flow rate of 10.0 mL/min. The retention times of compounds **1**–**4** were 16.31, 17.35, 26.25 and 28.00 min, respectively.

Identification of compounds 1–4

Compound 1 White amorphous powder; UV λ_{max} (MeOH) nm: 222, 238, 294, 334; IR ν_{max} (KBr) cm^{-1}: 3570 (NH), 3436 (br) (OH), 1600–1530 (CC, Ar), 1460–1380 (CC); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.75 (1H, s, NH), 7.64 (1H, d, J = 2.2 Hz, H-7), 7.63 (1H, dd, J = 8.4, 2.2 Hz, H-9) 6.69 (1H, d, J = 8.4 Hz, H-10), 6.81 (1H, s, H-6), 6.61 (1H, d, J = 9.9 Hz, H-1), 5.65 (1H, d, J = 9.9 Hz, H-2), 5.10 (1H, m, H-3′), 4.79 (1H, s, OH), 2.38 (3H, s, 5-Me), 2.15 (2H, m, H-2′), 1.75 (2H, m, H-1′), 1.65 (3H, s, H-6′), 1.57 (3H, s, H-5′), 1.44 (3H, s, H-7′); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 152.03 (C, C-4a), 150.31 (CH, C-9), 150.31, (C, C-8), 139.20 (C, C-10a), 136.14 (C, C-11a), 131.69 (C, C-5), 128.83 (CH, C-1), 124.13 (CH, C-2), 119.44 (CH, C-7), 117.58 (C, C-6b), 117.30 (CH, C-6), 116.24 (C, C-6a), 109.31 (CH, C-3′), 104.61 (C, C-4′), 96.73 (CH, C-10), 78.16 (C, C-3), 40.73 (CH_2, C-2′), 25.87 (CH_3, C7′), 25.65 (CH_3, C-6′), 22.72 (CH_2, C-1′), 17.61 (CH_3, C-5′), 16.12 (CH_3, 5-Me); HRFAB-MS m/z: 347.4573 $[M+H]^+$ (calc. 347.4580 for $C_{23}H_{25}NO_2$).

Compound 2 White amorphous powder; UV λ_{max} (MeOH) nm: 222, 242, 294; IR ν_{max} (KBr) cm^{-1}: 3569 (NH) 3396 (br, OH), 3570, 1624–1630 (CC, Ar), 1462–1381 (CC); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.81 (1H, brs, NH), 7.54 (1H, s, H-6), 7.72 (1H, d, J = 8.4 Hz, H-8), 6.68 (1H, dd, J = 8.4, 2.1 Hz, H-7), 6.61 (1H, d, J = 2.1 Hz, H-10), 6.60 (1H, d, J = 9.9 Hz, H-1), 5.65 (1H, d, J = 9.9 Hz, H-2), 5.11 (1H, m, H-3′), 4.89 (1H, brs, OH), 2.32 (3H, s, 5-Me), 2.16 (2H, m, H-2′), 1.75 (2H, t, J = 7.8 Hz, H-1′), 1.66 (3H, s, H-6′), 1.58 (3H, s, H-5′), 1.44 (3H, s, H-7′); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 153.48 (C, C-4a), 148.98 (CH, C-8), 140.71 (C, C-10a), 134.74 (C, C-11a), 131.65 (C, C-5), 128.72 (CH, C-1), 124.19 (CH, C-2), 120.39 (C, C-9), 119.98 (CH, C-7), 118.29 (C, C-11b), 118.11 (C, C-6b), 117.46 (CH, C-6), 116.68 (C, C-6a), 108.31 (CH, C-3′), 104.31 (C, C-4′), 96.94 (CH, C-10), 78 (C, C-3), 40.69 (CH_2, C-2′), 25.74 (CH_3, C-7′), 25.65 (CH_3, C-6′), 22.72 (CH_2, C-1′), 17.56 (CH_3, C-5′), 16.01 (CH_3, 5-Me); HRFAB-MS m/z: 347.4562 $[M+H]^+$ (calc. 347.4580 for $C_{23}H_{25}NO_2$).

Compound 3 Slight yellowish-green powder; UV λ_{max} (MeOH) nm: 224, 242, 304; IR ν_{max} (KBr) cm^{-1}: 3573 (NH), 1611–1530 (CC, Ar), 1461–1390 (CC); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.85 (1H, brs, NH), 7.75 (1H, dd, J = 8.4, 0.6 Hz, H-6), 6.73 (1H, dd, J = 8.4, 0.6 Hz, H-5), 7.72 (1H, s, H-7), 7.27 (1H, d, J = 8.1 Hz, H-10), 7.14 (1H, dd, J = 8.1, 1.2 Hz, H-9), 6.65 (1H, d, J = 9.9 Hz, H-1), 5.66 (1H, d, J = 9.9 Hz, H-2), 5.11 (1H, m, C-3′), 2.50 (3H, s, 8-Me), 2.15 (2H, m, H-2′), 1.75 (2H, m, H-1′), 1.65 (3H, s, H-5′), 1.57 (3H, s, H-6′), 1.45 (3H, s, H-7′); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 151.69 (C, C-4a), 137.71 (C, C-10a), 136.57 (C, C-11a), 131.71 (C, C-4′), 129.01 (C, C-8), 128.65 (CH, C-2), 125.73 (CH, C-9), 124.12 (C, C-6b), 124.12 (CH, C-3′), 120.38 (CH, C-6), 119.45 (CH, C-7), 117.31 (CH, C-1), 117.31 (C, C-6a), 110.06 (CH, C-10), 109.43 (CH, C-5), 104.51 (C, C-11b), 78.3 (C, C-3), 40.79 (CH_2, C-1′), 25.94 (CH_3, C-7′), 25.65 (CH_3, C-6′), 22.72 (CH_2, C-2′), 21.42 (CH_3, 8-Me), 17.61 (CH_3, C-5′); HRFAB-MS m/z: 331.4571 $[M+H]^+$ (calc. 331.4590 for $C_{23}H_{25}NO$).

Compound 4 Slight greenish-yellow powder; UV λ_{max} (MeOH) nm: 238, 286, 328; IR ν_{max} (KBr) cm^{-1}: 3530 (NH), 1610–1521 (CC, Ar), 1463–1389 (CC); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 7.91 (1H, d, J = 4.8 Hz, H-7), 7.88 (1H, s, NH), 7.67 (1H, s, H-6), 7.37 (1H, brd, J = 10.5 Hz, H-10), 7.31 (1H, dt, J = 7.2, 1.2 Hz, H-8), 7.18 (1H, dt, J = 7.2, 1.2 Hz, H-9), 6.65 (1H, d, J = 9.9 Hz, H-1), 5.66 (1H, d, J = 9.9 Hz, H-2), 5.12 (1H, tt, J = 4.2, 1.2 Hz, H-3′), 2.34 (3H, s, 8-Me), 2.18 (2H, m, H-2′), 1.77 (2H, dd, J = 9.3, 0.2 Hz, H-1′), 1.66 (3H, s, H-7′), 1.58 (3H, s, H-6′), 1.45 (3H, s, H-5′); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 149.9 (C, C-4a), 139.4 (C, C-10a), 134.8 (C, C-11a), 131.7 (C, C-4′), 128.5 (CH, C-2), 124.2 (CH, C-9), 124.2 (CH, C-3′), 123.9 (C, C-6b), 121.2 (CH, C-6), 119.4 (CH, C-8), 119.3 (CH, C-7), 118.4 (C, C-5), 117.5 (CH, C-1), 116.6 (C, C-6a), 110.4 (CH, C-10), 104.2 (C, C-11b), 78.1 (C, C-3), 40.8 (CH_2, C-1′), 25.8 (CH_3, C-7′), 25.6 (CH_3, C-5′), 22.7 (CH_2, C-2′), 17.6 (CH_3, C-6′), 16.1 (CH_3, 8-Me); HRFAB-MS m/z: 331.4571 $[M+H]^+$ (calc. 331.4590 for $C_{23}H_{25}NO$).

Pancreatic cholesterol esterase inhibitory activity

A modification of the method detailed by Gururaja et al. (2015) was used. Fractions and isolated compounds were prepared in ethanol as a stock solution (2 mg/mL) and diluted to 0.2 mg/mL with deionized water. Twofold serial dilutions were performed for IC_{50} determination. The final proportion of ethanol in the reaction was 2%. Pancreatic cholesterol esterase solution (2 µg/mL) was dissolved in 100 mM sodium phosphate buffer (pH 7.0). Various concentrations of the tested substances (100 µL) were pre-incubated with 50 µL of pancreatic cholesterol esterase solution at 37 °C for 15 min. *p*-Nitrophenyl butyrate (*p*-NPB), a substrate, was used to assay enzyme activity. The substrate solution was combined with a sodium phosphate buffer (0.1 M, pH 7.0) containing 1 volume each of 5.16 mM taurocholic acid sodium salt, 0.2 mM *p*-NPB, and 100 mM NaCl. After pre-incubation, the reaction was initiated by adding 50 µL of substrate solution to the mixtures, which were then

continuously incubated at 37 °C for 15 min. The reaction was stopped with the addition of 100 µL of ethanol. After incubation, the absorbance of the mixtures was measured at 405 nm. Orlistat and simvastatin were used as positive controls.

Method validation and quantification of pyranocarbazole alkaloids

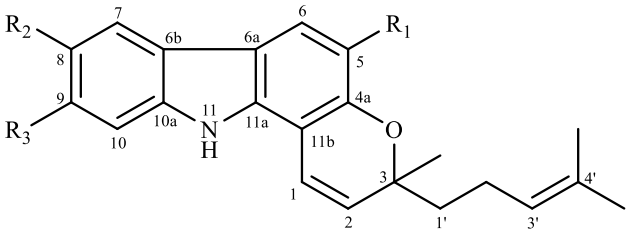
HPLC analysis of four pyranocarbazole alkaloids was carried out using the Agilent 1200 series with a 1200 quaternary HPLC pump and Agilent 1200 series Photodiode-array detector (PDA). The chromatographic separation was conducted using an Agilent Poroshell 120 PFP column (4.6 × 50 mm, 2.7 µm) with an isocratic solvent system of methanol and water (80:20 v/v) at room temperature (25 °C). The mobile phase flow rate and injection volume were 1 mL/min and 20 µL, respectively. The chromatograms were recorded at 285 nm for 15 min and data analysis was performed using OpenLab CDS EZChrom software.

Standard solutions were prepared by dissolving four pyranocarbazole alkaloids in isopropanol to prepare various concentrations (440–19 µg/mL). The dried plant powders (100 mg) were extracted with ethanol (10 mL) in an ultrasonic bath for 30 min. The extract solutions were filtered through filter paper and then evaporated to dryness under reduced pressure using a rotary evaporator. The dried residue was reconstituted and the volume adjusted to 5 mL with isopropanol. All sample solutions were filtered through a nylon filter (0.45 µM, CNW Technologies) prior to HPLC analysis. The contents of pyranocarbazole alkaloids in the sample were determined from the corresponding calibration curves. The HPLC–DAD method was validated through accuracy, intra-day and inter-day precision, specificity, the limit of determination (LOD) and limit of quantitation (LOQ), in accordance with the International Conference on Harmonization (ICH) guidelines.

Results and discussion

Systematic bioassay-guided isolation was conducted using an inhibitory enzyme bioassay to isolate and identify compounds extracted from *C. cambodiana* leaves that inhibit pancreatic cholesterol esterase. The ethanolic extract of the *C. cambodiana* leaves was purified using silica gel vacuum column chromatography, a dianion HP-20 column, and a preparative RP-HPLC column, yielding the four pyranocarbazole alkaloids (Fig. 1).

Compound **1** was a white amorphous powder, and its molecular formula was deduced to be $C_{23}H_{25}NO_2$ from the HRFAB-MS ion peak at 347.4573 *m/z* $[M+H]^+$ (calc. 347.4501 for $C_{23}H_{25}NO_2$). The UV spectrum of compound **1**



	R_1	R_2	R_3
Compound 1	CH_3	OH	H
Compound 2	CH_3	H	OH
Compound 3	H	CH_3	H
Compound 4	CH_3	H	H

Fig. 1 Chemical structures of isolated compounds **1–4**

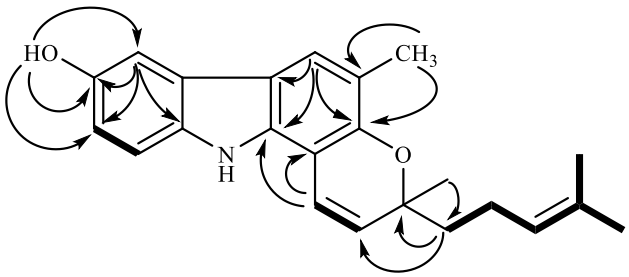


Fig. 2 Important HMBC (→) and COSY (▬) correlations of compound **1**

showed absorption maxima at 236, 264, and 312 nm, which are typical for the pyranocarbazole skeleton. Two singlets at δ 7.81 and 4.89 in the ^{1}H-NMR spectrum and IR absorption bands at 3570 and 3436 cm^{-1}, respectively, indicated the presence of –NH and phenolic hydroxyl functionalities. The ^{1}H and ^{13}C-NMR spectra of **1** showed signals assignable to two sp^2 methylenes (C-1′ and C-2′), four methyls (C-5a, C-5′, C-6′ and C-7′), seven sp^2 methines (C-1, C-2, C-3′, C-6, C-7, C-9 and C-10), nine sp^2 quaternary (C-4a, C-4′, C-5, C-6a, C-6b, C-8, C-10a C-11a and C-11b) and one sp^3 quaternary (C-3). The ^{1}H-NMR spectrum of **1** exhibited an ABX-type system at δ 7.64 (d, J = 2.2 Hz), 7.63 (dd, J = 8.4, 2.2 Hz), and 6.69 (d, J = 8.4 Hz). The signals of two *cis*-olefinic protons at δ 5.65 (d, J = 9.9 Hz) and 6.61 (d, J = 9.9 Hz), due to a pyran ring annulated to a carbazole moiety belong to H-1 and H-2. The ^{1}H-^{1}H COSY analysis of **1** (Fig. 2) led to the partial structural unit of 2-methylpent-2-en-5yl substituents at δ 1.44 (3H, s), 1.57 (3H, s), 1.65 (3H, s), 1.75 (2H, m), 2.15 (2H, m) and 5.10 (1H, m). The HMBC data (Fig. 2) propose the attachment of the 2-methylpent-2-en-5yl at the C-3 of the pyran ring and the hydroxyl group at C-8 of the aromatic ring. This data indicated that **1**

is a pyranocarbazole alkaloid that is similar to mahanine (**2**), except for the substituted position of the –OH group on the aromatic ring. These characteristics implied that compound **1** is 8-hydroxymahanimbine. Compound **1** has already been found in the literature as a synthetic compound from 2,6-dihydroxy-3-methylcarbazole (Anwer et al. 1972); however, it was isolated as a natural product for the first time.

Compound **2** had the same molecular formula as compound **1** ($C_{23}H_{25}NO_2$), based on its HRFAB-MS and ^{13}C-NMR spectra. The pyranocarbazole skeleton was deduced from the UV-absorption bands at λ_{max} 222, 242, 294, and 304 nm, which are typical for an angular pyranocarbazole moiety. The IR spectrum exhibited intense absorption bands at 3436 and 3540 cm^{-1}, suggesting the presence of –NH and –OH groups, respectively. The ^{1}H-NMR and ^{13}C-NMR spectra of **2**, similar to that of **1,** indicated the presence of 2-methylpent-2-en-5yl substituent in **2**. The location of substituents on the carbazolepyran skeleton of **2** was determined by COSY and HMBC measurements. Compound **2** was identified as mahanine by comparison with spectral data in the literature (Tachibana et al. 2001; Ramsewak et al. 1999; Reisch et al. 1992).

Compound **3** was isolated as a slightly yellowish-green powder. The molecular formula of compound **3** was assigned as $C_{23}H_{25}NO$ based on HRFAB-MS. The IR spectrum showed an absorption band of an –NH group at 3370 cm^{-1}. The ^{1}H-NMR, ^{13}C-NMR, and UV spectrum data for the pyranocarbazole nucleus were almost identical to those of compounds **1** and **2**. This data indicated that compound **3** is a pyranocarbazole alkaloid. Based on the above experimental data and a comparison of their NMR spectral data with published data (Tachibana et al. 2001), compound **3** was deduced to be mahanimbicine.

Compound **4** was obtained as a slightly greenish-yellow powder. The molecular formula of compound **4** ($C_{23}H_{25}NO$) was observed to be the same as compound **3**. The NMR data of **4** exhibited a pyranocarbazole nucleus in the molecule. The ^{1}H-NMR spectra at δ 7.91 (d, J = 4.8 Hz), 7.31 (dt, J = 7.2, 1.2 Hz), 7.18 (dt, J = 7.2, 1.2 Hz) and 7.37 (brd, J = 10.5 Hz) of **4** indicated an unsubstituted aromatic ring. Compound **4** was identified as mahanimbine by ^{1}H-NMR, ^{13}C-NMR, IR, and HRFAB-MS and compared with published data (Furukawa et al. 1985; Meragelman et al. 2000).

In the present study, we isolated and identified for the first time four pyranocarbazole alkaloids, 8-hydroxymahanimbine (**1**), mahanine, (**2**), mahanimbicine (**3**) and mahanimbine (**4**) from *C. cambodiana* leaves.

The four pyranocarbazole alkaloids isolated from *C. cambodiana* leaves were evaluated for their pancreatic cholesterol esterase inhibitory activity. The inhibitory activities of these four isolated compounds, orlistat, and simvastatin are shown in Table 1. 8-Hydroxymahanimbine (**1**) and mahanine (**2**) showed medium inhibitory activities against pancreatic cholesterol esterase, with IC_{50} values of 48.56 and 34.56 µg/mL, respectively, while mahanimbicine and mahanimbine were not active at a concentration of 100 µg/mL. These findings demonstrate that 8-hydroxymahanimbine and mahanine from *C. cambodiana* leaves seem to be potential inhibitors of pancreatic cholesterol esterase, which may result in reduced cholesterol absorption, and improved prevention and treatment of hypercholesterolemia.

Table 1 IC_{50} values of pyranocarbazole alkaloids from *C. cambodiana* leaves for anti-pancreatic cholesterol esterase activity

Samples	IC_{50}[a] (µg/mL)
Compound **1**	48.56 ± 0.45
Compound **2**	34.56 ± 0.23
Compound **3**	n.d.
Compound **4**	n.d.
Orlistat	0.35 ± 0.02
Simvastatin	5.73 ± 0.01

n.d. not determined at concentration 100 µg/mL

[a]Data are expressed as mean ± SD. For each sample n = 3

To control the quality of *C. cambodiana* leaves, the optimal conditions for the simultaneous quantitative determination of the biomarker compounds using the HPLC–DAD method were developed. In this study, while only compounds **1** and **2** exhibited an inhibitory effect against pancreatic cholesterol esterase activity, compounds **3** and **4** have been widely reported to show many biological effects, including cytotoxic activity, antibacterial activity and wound healing activity (Nagappan et al. 2011, 2012). Therefore, in this study, all pyranocarbazole alkaloids isolated from *C. cambodiana* leaves were used as biomarker compounds for quality control. The HPLC–DAD validated method was successfully applied for the simultaneous determination of four pyranocarbazole alkaloids in ethanol extracts from *C. cambodiana* leaves. The validation summary is shown in Table 2. The chromatographic separation of these compounds was achieved within 15 min with a satisfactory resolution (Fig. 3). All calibration curves for each constituent exhibited good linearity over the evaluated ranges (440–19 µg/mL) with correlation coefficients (r^2) of 0.9995. Intra-day and inter-day precisions were estimated by the percent relative standard deviation (%RSD), and were less than 2 and 5%, respectively. The percent recovery in the range of 95–101% was observed for all biomarker compounds. These values indicate the accuracy of the method. The specificity of the method was evaluated by comparing the retention time and the UV spectra of compounds **1**–**4** in the sample with the standard compounds. The retention times and UV-absorption spectra of compounds **1**–**4** in the sample were the same as the reference standards (Fig. 3). This result indicated that this method could be used for qualitative and quantitative

Table 2 The result of the validation of HPLC method for quantitative analysis of compounds **1**–**4**

Validation parameters	Compounds			
	1	**2**	**3**	**4**
Linearity				
Regression equation	$y = 1{,}651{,}238.29x + 444{,}545.29$	$y = 1{,}528{,}562.95x - 1{,}612{,}222.36$	$y = 2{,}184{,}437.13x + 23{,}631{,}576.25$	$y = 4{,}955{,}224.91x + 2{,}188{,}207.16$
R^2	1	0.9995	0.9996	0.9998
Linear range (μg/mL)	11.87–380	13.75–440	1.93–34.20	6.75–216
Retention time (min)	8.2	7.4	12.7	13.5
Precession (%RSD)				
Intra-day	0.87	0.56	1.17	0.79
Inter-day	1.89	1.65	2.24	1.76
Accuracy (% recovery)				
Level 1	99.72	101.82	96.23	97.56
Level 2	97.56	97.45	98.42	96.54
Level 3	97.48	95.97	97.66	97.90
LOD (μg/mL)	0.98	0.49	1.82	1.46
LOQ (μg/mL)	5.05	2.69	5.32	4.42

analysis of compounds **1**–**4**. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations: $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$. The LODs of compounds 1–4 were 0.98, 0.49, 1.82 and 1.46 μg/mL, respectively, and the LOQs of compounds **1**–**4** were 5.05, 2.69, 5.32 and 4.42 μg/mL, respectively (Table 2).

The pyranocarbazole alkaloid content in *C. cambodiana* leaves obtained from four different areas of Thailand in June 2019 was determined using the developed HPLC–DAD method. The contents of compounds **1**–**4** in *C. cambodiana* leaves are shown in Table 3. Mahanine (**2**) was present in the highest amount among the pyranocarbazole alkaloid (0.757 ± 0.036%w/w), followed by 8-hydroxymahanimbine (**1**) and mahanimbine (**4**) with contents of 0.176 ± 0.009 and 0.073 ± 0.004%w/w, respectively. Mahanimbicine (**3**) was present in the lowest amount (0.007 ± 0.001%w/w). The result suggested that this validated method could be applied for the quality control of *C. cambodiana* leaves in the future.

Conclusion

In summary, four pyranocarbazole alkaloids, 8-hydroxymahanimbine, mahanine, mahanimbicine and mahanimbine were isolated from *C. cambodiana* leaves for the first time through a bioassay-guided method. The isolated compounds in the series that demonstrated pancreatic cholesterol esterase inhibitory activity were 8-hydroxymahanimbine and mahanine. Besides, the HPLC–DAD method was established and validated for the determination of four pyranocarbazole alkaloids in *C. cambodiana* leaves. The precision, accuracy, specificity and sensitivity of this HPLC method were acceptable for the quantification of the pyranocarbazole alkaloids in *C. cambodiana* leaves. These results indicated that the developed HPLC analytical method can be utilized for the quality control of *C. cambodiana* leaves.

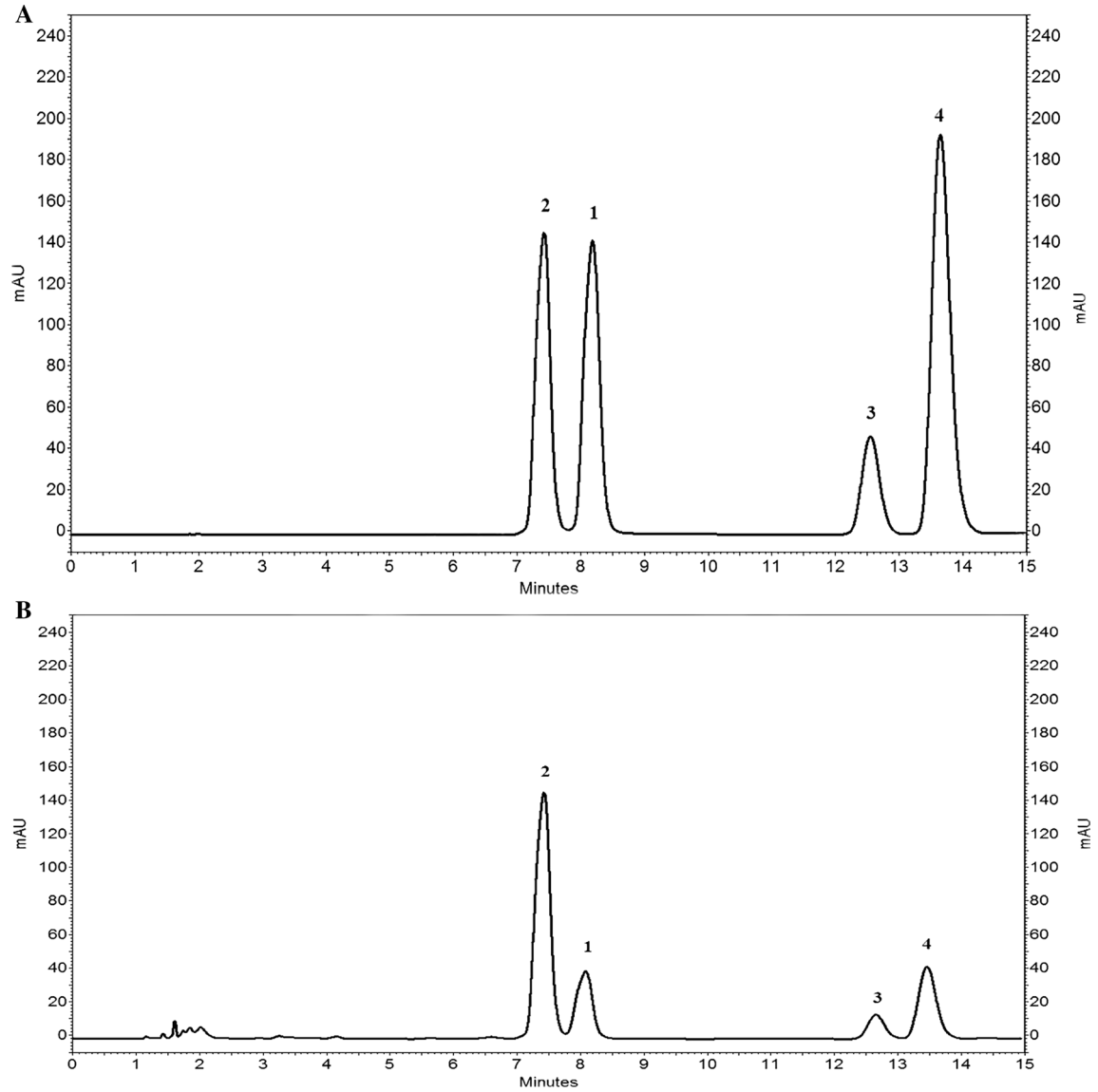


Fig. 3 HPLC chromatograms of standard compounds (**A**) 8-hydroxymahanimbine (**1**), mahanine (**2**), mahanimbicine (**3**), and mahanimbine (**4**) and ethanol extract of *C. cambodiana* leaves (**B**)

Table 3 Pyranocarbazole alkaloid contents in *C. cambodiana* leaves from different collection areas

Locations	Content (%w/w) [a]			
	1	**2**	**3**	**4**
Phatthalung Province	0.182 ± 0.026	0.712 ± 0.023	0.007 ± 0.001	0.077 ± 0.010
Trang Province	0.178 ± 0.016	0.782 ± 0.021	0.008 ± 0.001	0.071 ± 0.011
Songkhla Province	0.162 ± 0.023	0.743 ± 0.009	0.006 ± 0.001	0.068 ± 0.014
Yala Province	0.181 ± 0.019	0.790 ± 0.031	0.008 ± 0.002	0.075 ± 0.012
Averages	0.176 ± 0.009	0.757 ± 0.036	0.007 ± 0.001	0.073 ± 0.004

[a]Data are expressed as mean ± SD. For each sample $n = 3$

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Compliance with ethical standards

Conflict of interest There are no conflicts to declare.

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