ISOLATION OF β-SITOSTEROL AND DIGALACTOPYRANOSYL-DIACYLGLYCERIDE FROM *CITRUS HYSTRIX,* A THAI TRADITIONAL HERB, AS PANCREATIC LIPASE INHIBITORS

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Abstract – β-Sitosterol and 3-[*O*-α-galactopyranosyl-(1″→6′)-*O*-β-galactopyranosyl]-1-*O*-linolenyl-2-*O*-palmitylglyceride were isolated from the leaves of *Citrus hystrix,* a Thai traditional herb, as pancreatic lipase inhibitors.

INTRODUCTION

Obesity has been very serious problems in advanced countries because of high risk for life-style related diseases causing hypertension, diabetes mellitus, and dyslipidemia.[1]　A lipid, triacylglyceride, can be absorbed after hydrolysis to free fatty acids and glycerols with lipases, especially pancreatic lipase. Thus, lipase inhibitors could be promising candidates for an improvement of corpulent states.[2]　Orlistat (XENICAL[®]) has been used as a synthetic pancreatic triacylglyceride lipase inhibitor for obesity management that acts by inhibiting the absorption of the dietary triacylglycerides.　We have explored pharmacologically active and useful seeds sources from Thai medicinal plants[3] and targeted on the isolation of anti-pancreatic lipase active components from the leaves of *C. hystrix* DC.　In this paper we report the identification of β-sitosterol and 3-[*O*-α-galactopyranosyl-(1″→6′)-*O*-β-galactopyranosyl]-1-*O*-linolenyl-2-*O*-palmitylglyceride as active principles based on activity-guided separation.

RESULTS AND DISCUSSION

Identification of pancreatic lipase inhibitors from natural sources have been extensively studied;[4] for example, it has been reported the isolation of naphthylglucosides from *Juglans mandshurica*,[5] polyphenols from *Salacia reticulata*,[6] and ginseng saponins from *Panax ginseng*[7] as plant-derived pancreatic lipase inhibitors.　Screening ethanol extracts prepared from 44 Thai plants showed that inhibition activity (> 50% at the concentration of 100 µg/mL) was observed in the 5 extracts derived from *Adenanthea pavonina* (No. 14; 51%), *Citrus hystrix* (No. 21; 58%), *Michelia alba* (No. 23; 70%), *Gustavia gracillima* (No. 25; 56%), and *Phyllanthus acidus* (No. 27; 63%) (Figure 1).　Among them *C. hystrix* was selected as a target plant at the next stage for the isolation of active principle(s) because of one of the most common and important Thai traditional herb, especially one of spices sources for famous Thai cosine "TOM YAM KUNG," in addition to showing a variety of biological activities;[8] for example, on the leaves, antimicrobial activity,[8a,8b] cytotoxic activity,[8b] inhibition of nitric oxide production,[8c,8d] effect for chronic arthritis,[8d] antioxidant activity,[8e] and suppression of tumor promotion[8f] are known.

Activity-guided separation of the ethanol extract of the leaves of *C. hystrix* using chromatographic techniques resulted in the isolation of two active components.　The isolate from less polar fraction was identified to be β-sitosterol, which showed 79.1±11.3% inhibition activity at the concentration of 100 µg/mL and has been known as anti-pancreatic active principle.[9]

The isolate from more polar fraction showed stronger inhibition (88.2±2.5% at the same concentration) than β-sitosterol and was suggested to be a diacylglycolipid derivative by spectroscopic analysis.
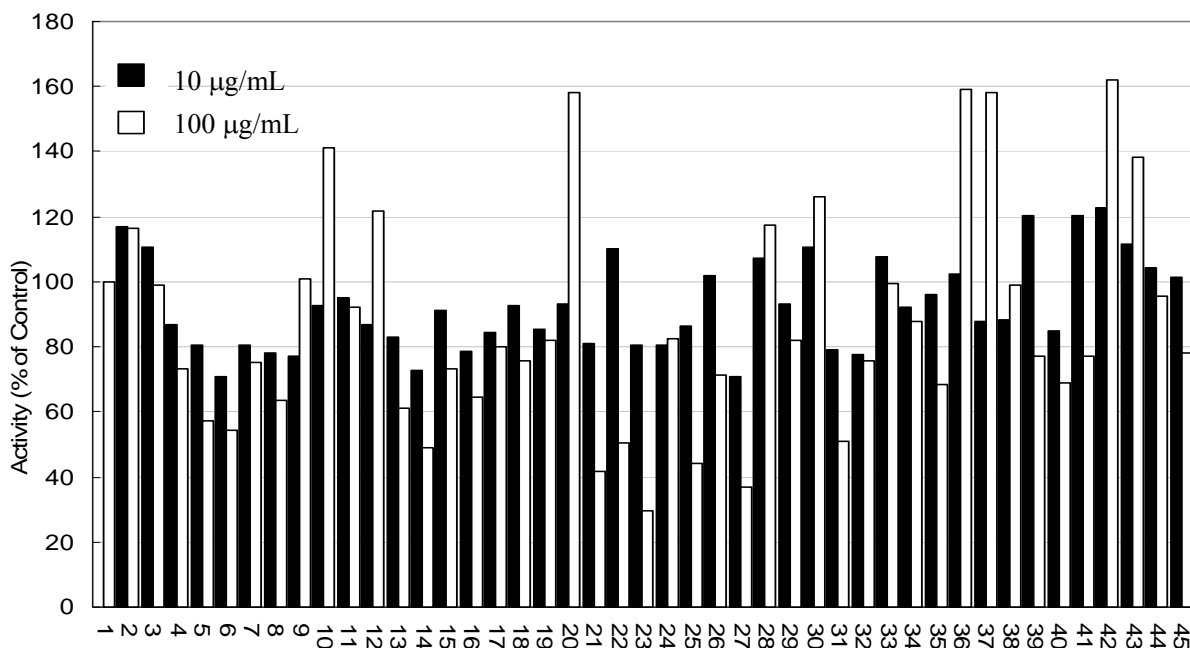
1: control
2: *Ocimum basilicum* (Labiatae)
3: *Pterocarpus indicus* (Leguminosae)
4: *Arfeuillea arborescens* (Sapindaceae)
5: *Cratoxylum formosum* (Guttiferae)
6: *Polygonum odoratum* (Polygonaceae)
7: *Oxyceros horridus* (Rubiaceae)
8: *Garcinia cowa* (Guttiferae)
9: *Ocimum tenuiflorum* (Labiatae)
10: *Morinda citrifolia* (Rubiaceae)
11: *Centella asiatica* (Umbelliferae)
12: *Lagerstroemia speciosa* (Lythraceae)
13: *Murraya paniculata* (Rutaceae)
14: *Adenanthera pavonina* (Leguminosae)
15: *Acacia pennata willd. subsp. insuavis* (Leguminosae)
16: *Glycosmis pentaphylla* (Rutaceae)
17: *Sesbania grandiflora* (Leguminosae)
18: *Foeniculum vulgare* (Umbelliferae)
19: *Alangium salviifolium subsp. hexapetalum* (Alangiaceae)
20: *Momordica charantia* (Cucurbitaceae)
21: *Citrus hystrix* (Rutaceae)
22: *Careya sphaerica* (Lecythidaceae)

23: *Michelia alba* (Magnoliaceae)
24: *Mitragyna hirsuta* (Apocynaceae)
25: *Gustavia gracillima* (Lecythidaceae)
26: *Tectona grandis* (Verbenaceae)
27: *Phyllanthus acidus* (Euphorbiaceae)
28: *Aegle marmelos* (Rutaceae)
29: *Piper sarmentosum* (Piperaceae)
30: *Alstonia scholaris* (Apocynaceae)
31: *Thunbergia laurifolia* (Acanthaceae)
32: *Atalantia monophylla* (Rutaceae)
33: *Tamarindus indicus* (Leguminosae)
34: *Leucaena leucocephala* (Leguminosae)
35: *Bridelia ovata* (Euphorbiaceae)
36: *Hymenodictyon orixense* (Rubiaceae)
37: *Cassia glauca* (Leguminosae)
38: *Monochoria vaginalis* (Pontederiaceae)
39: *Anethum graveolens* (Umbelliferae)
40: *Averrhoa bilimbi* (Averrhoaceae)
41: *Sechium edule* (Cucurbitaceae)
42: *Myristica fragrans* (Myristicaceae)
43: *Mansonia gagei* (Sterculiaceae)
44: *Cucurbita moschata* (Cucurbitaceae)
45: *Radermachera ignea* (Bignoniaceae)

Figure 1. Preliminary inhibitory activity of the ethanol extracts of 44 Thai plants against pancreatic lipase

Table 1. NMR data (δ) of the diacylglycolipid (CD_3OD)

#	^1H (600 MHz)	^{13}C (150 MHz)
CaH_3	0.86 (3H, t, J = 6.9 Hz)	14.7
CbH_3	0.94 (3H, t, J = 7.6 Hz)	14.9
CcH_2	1.20-1.40 (32H, broad s-like)	23.9, 30.3, 30.36, 30.41, 30.5, 30.6, 30.8, 30.88, 30.91, 31.0, 33.2
CdH_2	1.54-1.60 (4H, br)	26.16, 26.18
CeH_2	2.10-2.08 (4H, m)	21.7, 28.3
CfH_2	2.28 (2H, t, J = 7.5 Hz)	35.1
	2.29 (2H, t, J = 7.4 Hz)	35.3
CgH_2	2.77 (4H, t, J = 5.9 Hz)	26.6, 26.7
C3'H	3.46 (1H, dd, J = 9.6, 3.1 Hz)	72.5
C2'H	3.48 (1H, t, J = 9.6 Hz)	74.8
C6'H_2	3.64 (1H, dd, J = 10.2, 6.1 Hz)	67.9
	3.84-3.88* (1H, m)	
C6"H_2	3.67-3.73* (2H, m)	62.9
C3"H	3.71 (1H, dd, J = 10.3, 3.8 Hz)	71.6
C5'H	3.67-3.73* (1H, m)	74.6
C3H_2	3.67-3.73* (1H, m)	68.9
	3.90 (1H, dd, J = 10.9, 5.4 Hz)	
C2"H	3.75 (1H, dd, J = 10.1, 3.7 Hz)	70.3
C5"H	3.82 (1H, t, J = 6.2 Hz)	72.6
C4'H	3.86 (1H, d, J = 3.6 Hz)	70.1
C4"H	3.84-3.88* (1H, m)	71.2
C1H_2	4.20 (1H, dd, J = 12.1, 6.9 Hz)	64.2
	4.40 (1H, dd, J = 12.1, 2.8 Hz)	
C1'H	4.22 (1H, d, J = 7.1 Hz)	105.4
C1"H	4.84 (1H, d, J = 3.8 Hz)	100.7
C2H	5.20-5.36* (1H, m)	71.9
CkH	5.20-5.36* (6H, m)	128.3, 129.0, 129.3, 129.4, 131.2, 132.9
CO	-	174.9, 175.3

 * overlapped

It was obtained as an optically-active colorless oil and its molecular formula was deduced to be $C_{49}H_{86}O_{15}$ (914) from FAB and MALDITOF mass spectra. The IR spectrum showed the presence of hydroxyl and carbony groups, which were attributable to sugar and fatty acid ester functions, respectively, based on the analysis of the NMR spectra (Table 1). The sugar function was assignable to be a diglycoside (the carbon sequences of C1'- C6' and C1"- C6") due to the appearances of 14 protons at δ 3.46-4.84 in the ^1H NMR spectrum and of 12 signals in the range of δ 62.9-100.7 in the ^{13}C NMR spectrum. The fatty acid ester (the carbon sequences of Ca-Cg, Ck, and CO) was suggested to be composed of two long-chain acids, including a 1,4,7-triene system, due to the appearances of 54 protons at δ 0.86-2.77 and 6 protons at δ 5.20-5.36 in the ^1H NMR spectrum and the geometry of the triene system is assigned to be a *cis-cis-cis* sequence due to the appearance of all the allylic carbons (Ce and

Cg) at δ around 28.[10]   The presences of 21, 6, and 2 signals in the range of δ 14.7-35.3, 128.3-132.9, and around 175, respectively, even overlapping some signals, in the ^{13}C NMR spectrum reasonably supported the above assignment.   The remaining 5 protons at δ 3.67-3.73 (1H, m), 3.90 (1H, dd, J = 10.9, 5.4 Hz), 4.20 (1H, dd, J = 12.1, 6.9 Hz), 4.40 (1H, dd, J = 12.1, 2.8 Hz), and 5.20-5.36 (1H, m) in the ^{1}H NMR and 3 signals at δ 64.2, 68.9, and 71.9 in the ^{13}C NMR spectrum were assigned to be glycerol function (the carbon sequence of C1- C3).
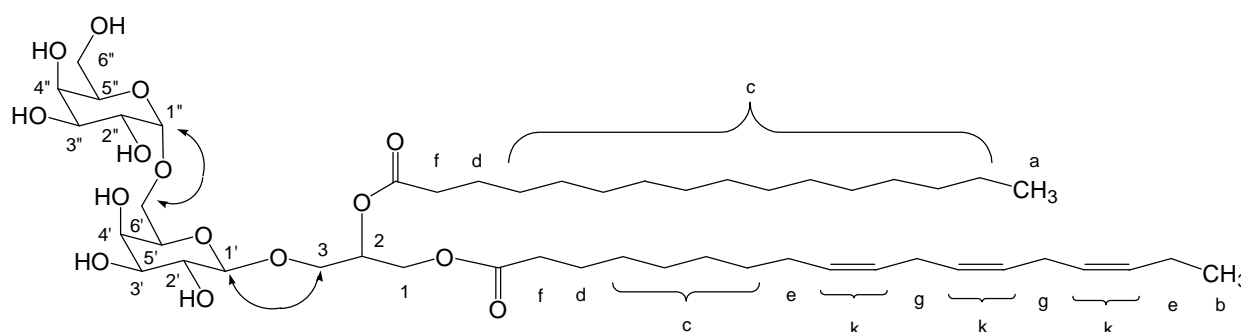

Figure 2.   Selected HMBC correlation and structure of the diacylglycolipid

Precise examination of 2D NMR (COSY, HMQC, HMBC, and TOCSY) techniques including HSQC experiment ([1]J coupling constants of the anomeric positions: 160 Hz for C1'H, 170 Hz for C1"H)[11] allowed us to deduce the structure of the diglycoside to be O-α-galactopyranosyl-(1"→6')-O-β-galactopyranoside, which was bonded to the primary alcohol of the glycerol unit.   Further analysis of 2D NMR experiments suggested the presence of palmitic and linolenic acids as fatty acid units.   Overlapping the CfH$_2$ signals of fatty acid esters in the ^{1}H NMR spectrum could not allow us to discriminate their mode of connection to the glycerol function; however, easier hydrolytic removal (under keeping in a protic solvent at room temperature) of the unsaturated acid unit strongly suggested the possible connection of the linolenic acid unit to the primary alcohol function of the glycerol.   Thus, the combination of three partial structures (sugar, fatty acid, and glycerol units) led to the deduction of the isolate to be 3-[O-α-galactopyranosyl-(1"→6')-O-β-galactopyranosyl]-1-O-linolenyl-2-O-palmitylglyceride[12] (Figure 2).

CONCLUSION

In conclusion, β-sitosterol and 3-[O-α-galactopyranosyl-(1"→6')-O-β-galactopyranosyl]-1-O-linolenyl-2-O-palmitylglyceride were isolated from the leaves of *Citrus hystrix* as pancreatic lipase inhibitors.   Although the structurally related galactopyranosyl-dilinolenylglyceride and

galactopyranosyl-linolenyl-palmitoylglyceride have been isolated from *C. hystrix*[8f] as inhibitors against cancer promotion, there have been no reports on the isolation of digalactopyranosyl-diacylglyceride from the same plant.　　Interestingly, it was recently reported that 3-β-D-galctopyranosyl-1-*O*-palmitonyl-2-*O*-caprylonylglyceride isolated from *Spirulina platensis* showed inhibitory effect against pancreatic lipase (*ca* 70% inhibition at the concentration of 100 μg/mL).[13]　　Thus, our result is not only the first isolation of 3-*O*-digalactopyranosyl-1-*O*-linolenyl-2-*O*-palmitylglyceride from *C. hystrix*, but also the new finding of its inhibitory effect on pancreatic lipase (*ca* 90% inhibition at the concentration of 100 μg/mL).　　In other words, the leaves of *C. hystrix*, a Thai traditional herb, could be a potential and natural dietary candidate for improving obesity.

EXPERIMENTAL

General experimental procedures.　　Melting points were determined on a micromelting point hot-stage apparatus (Yanagimoto) and are uncorrected.　　IR spectra were recorded on a JASCO IR-300 E spectrophotometer.　　Optical rotation ($[\alpha]_D$) was recorded on a JASCO P-1020 polarimeter.　　^{1}H and ^{13}C NMR spectra were recorded with JEOL JNM ECA 600 and ECP 600 spectrometers with TMS as an internal reference.　　FAB and MALDITOF mass spectra were measured on a JEOL JMS-AX500 and an Applied Biosystems Voyager DE-PRO (Framingham) mass spectrometers, respectively.　　Instrument calibration in MALDITOF mass spectrum was performed with external standards using synthetic enolase phosphopeptides (Waters).　　For column chromatography and TLC, Si gel 60 (70–230 mesh ASTM; Merck) and Si gel 60 F254 (Merck) were used, respectively.　　A Biotage C18HS 12+M column was used for reverse-phase flash chromatography.　　For recycle HPLC was used LC-9201 (Japan Analytical Industry Co. Ltd).

Measurement of anti-pancreatic lipase activity in vitro.　　For the preliminary screening assay of pancreatic lipase activity, the following method was employed using pig pancreatic lipase adjusted at 1.5 u/30 mM Tris buffer.　　Intrapolis (Otsuka Pharmaceutical Co.) containing 1.2% yolk lectin, 2.5% glycerol, and 10% soybean oil was used as a substrate.　　A mixture of substrate (25 μL), 30 mM Tris·HCl buffer (pH 8.5) (150 μL), 300 mM Tris·HCl buffer (pH 8.5) (50 μL), 1% cholic acid (50 μL), and 1 mM CaCl$_2$ (25 μL) was added each plant extract (2 μL) and then pre-incubated at 37 °C for 30 min.　　After addition of the enzyme solution (200 μL) the whole was incubated at 37 °C for 30 min and then quenched with copper reagent (0.45 M triethanolamine, 0.05 M acetic acid, 3.4% CuSO$_4$· 5H$_2$O, and 20% NaCl) (1 mL).　　The liberated fatty acid component(s) was partitioned with a mixture of CHCl$_3$ and *n*-heptane (4 : 6) (3 mL) and centrifuged (3000 rpm for 7 min).　　To the organic layer separated was

added coloring reagent [0.1% bathocuproine, and 0.05% butylhydroxyanisole/CHCl$_3$ and n-heptane (4 : 6)] (2 mL) and absorbance at 480 nm was measured.   The same 30 mM Tris·HCl buffer (pH 8.5) and dimethyl sulfoxide (DMSO) were used as blank instead of enzyme and control instead of extract, respectively.

For further assay in activity-guided separation, the method of Slanc *et al.*[14] was employed with some modifications.   *p*-Nitrophenyl palmitate (PNP) was dissolved in mixed solvent (MeCN : EtOH = 3 : 1) to the concentration of 3.33 mM PNP and stored at -30 ℃.   A test sample examined was dissolved in DMSO (1 mg/mL).   Porcine pancreatic lipase (Sigma: type II, 100-400 u/mg) was dissolved in 75 mM Tris·HCl buffer (pH 8.5) to the concentration of 0.5 mg/mL.   A mixed solution of the sample (10 μL), 3.33 mM PNP (10 μL), 75 mM Tris·HCl buffer (pH 8.5) (40 μL), and the enzyme (40 μL) was incubated at 37 ℃ for 25 min.   After addition of MeCN (100 μL) a mixture was agitated carefully. The absorbance of released *p*-nitrophenol was measured at 405 nm using a microplate reader.   The enzyme was replaced by 75 mM of Tris·HCl buffer (pH 8.5) and the extract by DMSO for the blank and the control, respectively.   A solution of Orlistat in DMSO was also used at the final concentration of 100 ng/mL as a positive control.   Absorbance of samples was corrected by subtracting the absorbance of blanks.   The difference between the enzyme activity of the control and the sample was defined as the inhibitory activity of the extract.   The activity assay was performed in triplicate for each plant extract. The results were averaged and expressed with standard deviations (SD).   Relative large SD (*ca* 10%) were observed even the use of acetonitrile (10 μL) as a co-solvent in place of one forth of Tris·HCl buffer (pH 8.5) for dissolving a test sample.

Source of plant material   The leaves of *C. hystrix* DC. were collected in Nonthaburi province in the central part of Thailand in 2007.   Voucher herbarium specimens (No. 086254) of the plant were identified and deposited at the Forest Herbarium, National Park Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment, Bang Khen, Bangkok, Thailand.

Extraction and isolation.   As a preliminary trials for the identification of active components, the methanol soluble fraction (65.7 g) of ethanol extract (72.8 g) prepared from the leaves of *C. hystrix* (473 g) was subjected to activity-guided fractionation (> 60% inhibitory activity at the concentration of 100 μg/mL) by column chromatography using the graduation system of EtOAc and hexane (32 : 1, 16 : 1, and 1 : 1) to give less polar (3.75 g, eluted with hexane : EtOAc = 16 : 1) and more polar active fractions (13.2 g, eluted with hexane : EtOAc = 1 : 1).   A part of the less polar fraction (135 mg) was further purified by column chromatography using the same graduation system of solvent (16 : 1, 10 : 1, 8 : 1,

and 4 : 1).   The fraction obtained in hexane : EtOAc = 10 : 1 was purified by recycle HPLC (CHCl₃) followed by recrystallization (EtOAc-MeOH) to give β-sitosterol [6 mg (calculated to 161 mg), 0.03%] as an active component (20.9±11.3% activity of control).   A large amount of plant material (3 kg) was used for the separation of more polar component(s) because the isolation of active principle(s) from the more polar fraction obtained above was failed.   Thus, newly prepared ethanol extract (481 g) was at first partitioned with EtOAc (315 g) and water (167 g).   A part of the ethyl acetate solution (110 g) was separated to 6 fractions by column chromatography (CHCl₃ : MeOH = 100 : 1, 15 : 1, 10 : 1, 5 : 1, 1 : 1, and 0 : 1).   A part (3.12 g) of the fifth fraction (9.82 g) was subjected to reverse-phase flash chromatography (MeOH : MeCN = 4 : 1) to give 6 fractions, among which the fifth fraction (253 mg) was further purified by reverse-phase flash chromatography (MeOH : MeCN : water = 40 : 10 : 1) to give a diacylglycolipid [188 mg (calculated to 1.84 g), 0.06%] as an alternative active principle (11.8± 2.5% activity of control).

3-[*O*-α-Galactopyranosyl-(1"→6')-*O*-β-galactopyranosyl]-1-*O*-linolenyl-2-*O*-palmitylglyceride. Colorless oil, IR (ATR) ν_{max} 3381 1732 cm⁻¹; ¹H- and ¹³C-NMR, see Table 1; FABMS *m/z*: 973 (M+Na)⁺; MALDITOFMS *m/z*: 973.7137 (M+Na)⁺; $[\alpha]_D^{19.4}$ +37.4° (*c* 0.6, MeOH).

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