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Anti-allergic substances from the rhizomes of *Dioscorea membranacea*

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Abstract—Extracts of five species of Thai medicinal plants, locally known as Hua-Khao-Yen, were screened for anti-allergic activities using RBL-2H3 cells. Of the five species studied, the ethanolic extract of *Dioscorea membranacea* exhibited potent inhibitory activity against β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells, with an IC_{50} value of 37.5 μg/mL. Eight compounds were isolated from this crude ethanolic extract, [two naphthofuranoxepins (**1**, **2**), one phenanthraquinone (**3**), three steroids (**4**–**6**), and two steroidal saponins (**7**, **8**)], and tested for their anti-allergic activities. The results showed that dioscorealide B (**2**) possessed the highest activity with an IC_{50} value of 5.7 μM, followed by dioscoreanone (**3**, IC_{50} = 7.7 μM), dioscorealide A (**1**, IC_{50} = 27.9 μM), and diosgenin (**9**, IC_{50} = 29.9 μM). Structure–activity relationship studies of naphthofuranoxepins on anti-allergic activity revealed that the hydroxylation at position 8 conferred higher activity than methoxylation. For diosgenin derivatives, the aglycone was found to possess higher activity than the diglucosylated molecule; whereas substitution with rhamnoglucosides apparently results in loss of activity. Furthermore, effects of dioscorealide A, dioscorealide B, and dioscoreanone on antigen-induced release of TNF-α and IL-4 in the late phase reaction were also examined.

1. Introduction

Nowadays, there are many drugs available as antihistamine and anti-allergic agents, however, they have undesirable side effects and adverse reactions, such as drowsiness, headache, gastrointestinal tract disturbance, fatigue, and dry mouth. Since the modern medicines have some limited use, the traditional herbs are now becoming the promising approach for the treatment of allergies.

The allergy is an immune dysfunction, which is a serious health problem worldwide. Substances that cause allergic reaction are called allergens including food, pollen, dust mites, cosmetics, mold spores, and animal hairs. Hypersensitivity type I, an allergic reaction, is an IgE-mediated immune response, resulting in histamine secretion from mast cells and blood basophils. The histamine causes smooth muscle contraction, increased vascular permeability, and vasodilation. The early phase reaction of allergy occurs within minutes after allergen exposure, whereas the late phase reaction occurs hours later and involves in cytokines' secretion such as TNF-α and IL-4.[1] Since, β-hexosaminidase is usually released along with histamine from mast cells or basophils, this enzyme is therefore used as the marker for mast cell degranulation in RBL-2H3 cell line.[2]

In Thai traditional medicine, herbal drugs locally known as 'Hua Khao-Yen' have long been used as common ingredients in many preparations, including those used in treatments of dermopathy, lymphopathy, inflammation, cancers, veneral diseases, and leprosy. Hua-Khao-Yen is the name of traditional drugs that come from different plant species of at least three genera, *Dioscorea* from Disocoreaceae, *Smilax* of the Smilacaceae, and *Pygmaeopremna* of the Verbenaceae.[3]

Since the rhizomes of Hua-Khao-Yen have long been used by Thai traditional doctors for the treatment of dermopathy and lymphopathy, this study therefore examined the inhibitory activity of these plants against allergic reaction in a cell line model. In addition, we also reported the isolation and testing of eight compounds

Keywords: RBL-2H3 cells; Anti-allergic activity; *Dioscorea membranacea*.
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from the crude extract of *Dioscorea membranacea* and comment on the structure–activity relationships what might explain the activity profiles of the different compounds.

2. Results and discussion

Five species of Thai medicinal plants locally known as Hua-Khao-Yen were investigated for their anti-allergic activities using RBL-2H3 cells. Among these species, the ethanolic extract of *D. membranacea* exhibited appreciable activity with an IC_{50} value of 37.5 μg/mL. This plant extract was further subjected to silica gel column chromatography and HPLC-RP to isolate eight compounds (Fig. 1), two naphthofuranoxepins (**1**,**2**), one phenanthraquinone (**3**), three steroids (**4**–**6**), and two steroidal saponins (**7**,**8**). The result indicated that dioscorealide B (**2**) possessed the highest activity with an IC_{50} value of 5.7 μM, followed by dioscoreanone (**3**, IC_{50} = 7.7 μM), dioscorealide A (**1**, IC_{50} = 27.9 μM), and diosgenin (**9**, IC_{50} = 29.9 μM), respectively, whereas other compounds exhibited moderate and weak activities (Table 1). Structure–activity relationship studies of naphthofuranoxepins revealed that the hydroxylation at position 8 showed activity fivefold higher than the methoxylation, as observed in IC_{50} value of **2** (IC_{50} = 5.7 μM) versus that of **1** (IC_{50} = 27.9 μM). Moreover, the structural requirements of diosgenin and its derivatives for anti-allergic activity indicated that aglycone itself (**9**, IC_{50} = 29.9 μM) gave higher activity than glycosylation with diglucosides (**8**, IC_{50} = 68 μM) or rhamnoglucosides (**7**, IC_{50} > 100 μM). Compounds **1**–**9** were also examined on the enzyme activity of β-hexosaminidase. As a result, they showed weak inhibition against this enzyme activity at 100 μM (Table 1). The result indicated that these compounds inhibited the antigen-induced degranulation but not substantially affected the activity of β-hexosaminidase.

Dioscorealide A (**1**), dioscorealide B (**2**), and dioscoreanone (**3**) were also tested against antigen-induced TNF-α and IL-4 release, which participate in the late phase of type-I allergic reaction in RBL-2H3 cells. It was found that dioscoreanone (**3**) exhibited the most potent against TNF-α release with an IC_{50} value of 8.1 μM, followed by dioscorealide B (**2**, IC_{50} = 22.0 μM) and dioscorealide A (**1**, IC_{50} = 33.1 μM), respectively (Table 2). For an inhibition on IL-4 release, **3** again showed the highest inhibitory activity with an IC_{50} value of 6.0 μM, followed by **1** (IC_{50} = 36.2 μM) and **2** (IC_{50} = 73.6 μM). The result indicated that compound **2** exhibited stronger effect on the early phase reaction than the late phase one, whereas **1** and **3** possessed comparable activities in both phases (Table 2). This might reveal that the mechanism of action of compound **2** on the degranulation (the early phase reaction) is somewhat different from that on the release of TNF-α and IL-4 (the late phase reaction). Regarding bioactivities of *D. membranacea*, it has been reported that dioscorealide B (**2**) and

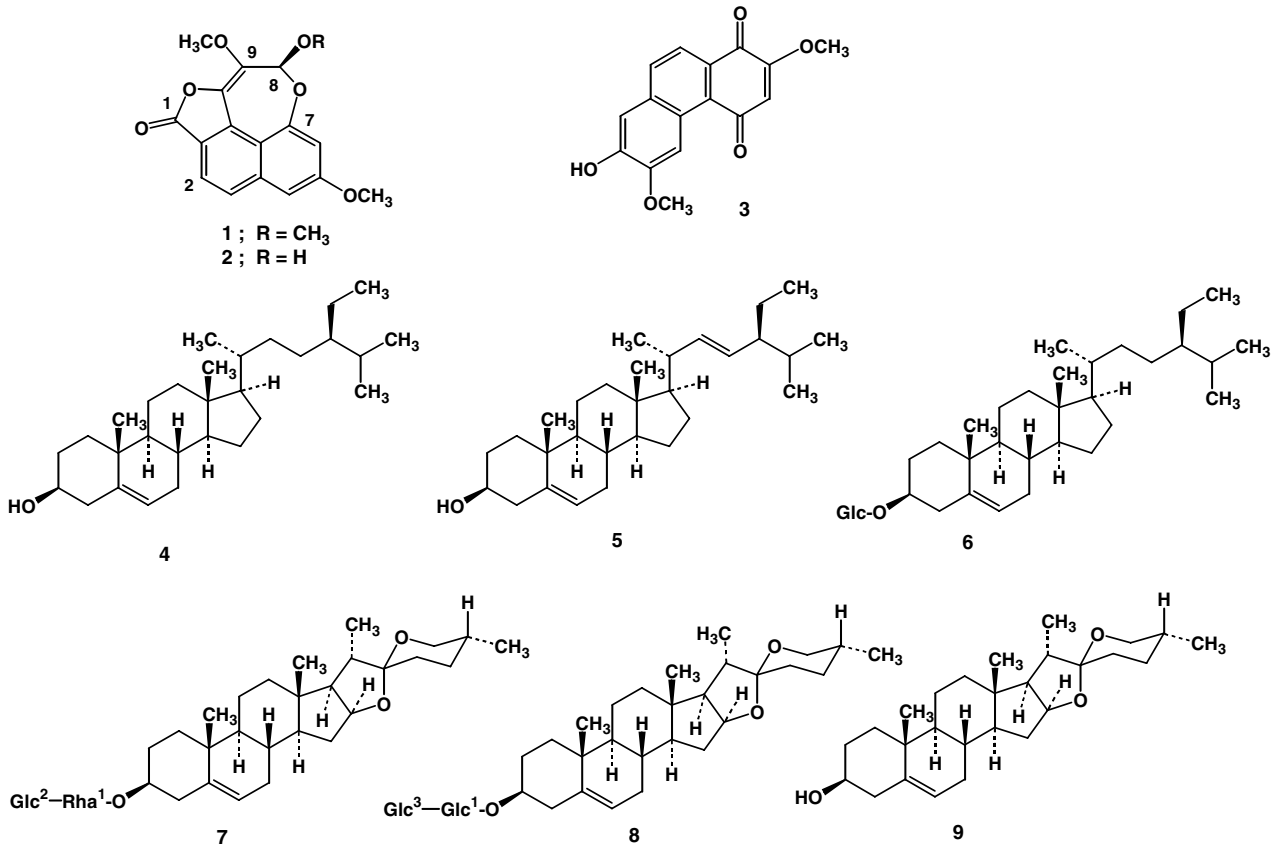

Figure 1. Chemical structures of compounds **1**–**9** isolated from *Dioscorea membranacea*.

Table 1. Anti-allergic activities of compounds from *Dioscorea membranacea* against antigen-induced β-hexosaminidase release from RBL-2H3 cells[a]

Compound	% Inhibition at various concentrations (μM)					IC_{50} (μM)	Enzyme inhibitory activity at 100 μM (%)
	0	3	10	30	100		
Dioscorealide A (**1**)	0.0 ± 5.2	—	−11.8 ± 7.5	79.0 ± 6.4**	97.9 ± 1.1**	27.9	18.5
Dioscorealide B (**2**)	0.0 ± 2.8	30.9 ± 1.6**	66.0 ± 9.6**	90.3 ± 9.7**	98.8 ± 0.9**	5.7	21.9
Dioscoreanone (**3**)	0.0 ± 7.0	9.2 ± 6.8	79.1 ± 6.4**	92.1 ± 1.6**	96.0 ± 0.4**	7.7	20.6
β-Sitosterol (**4**)	0.0 ± 7.9	—	9.9 ± 4.7	46.1 ± 4.3*	68.7 ± 2.2**	43.2	23.1
Stigmasterol (**5**)	0.0 ± 9.1	—	24.7± 8.6	46.9 ± 8.7*	64.8 ± 1.6**	40.3	21.6
β-Sitosterol-3-*O*-β-D-glucopyranoside (**6**)	0.0 ± 9.0	—	−13.0 ± 10.1	33.6 ± 6.8	62.0 ± 3.9**	62.1	20.2
Diosgenin-3-*O* − α-L-rhamnosyl (1 → 2)-β-D-glucopyranoside (**7**)	0.0 ± 6.4	—	—	—	−7.0 ± 1.6	>100	—
Diosgenin-3-*O*-β-D-glucopyranosyl (1 → 3)-β-D-glucopyranoside (**8**)	0.0 ± 4.2	—	−11.6 ± 5.8	37.1 ± 7.4**	56.0 ± 3.9**	68.0	20.2
Diosgenin (**9**)	0.0 ± 4.2	1.0 ± 4.5	36.2 ± 7.4*	44.2 ± 5.8**	75.1 ± 4.4**	29.9	25.5
Ketotifen fumarate	0.0 ± 5.9	—	12.8 ± 0.5	38.3 ± 3.2**	68.2 ± 1.5**	47.5	15.8

Statistical significance, $^{*}p < 0.05$, $^{**}p < 0.01$.
[a] Each value represents means ± SEM of four determinations.

Table 2. Inhibitory effects of compounds **1**–**3** of *Dioscorea membranacea* on antigen-induced TNF-α (A) and IL-4 (B) release from RBL-2H3 cells[a]

Compound	% Inhibition at various concentrations (μM) against TNF-α release					IC_{50} (μM)
	0	3	10	30	100	
(A)						
Dioscorealide A (**1**)	0.0 ± 3.8	—	−9.7 ± 3.5	56.0 ± 5.6**	95.1 ± 6.4**	33.1
Dioscorealide B (**2**)	0.0 ± 4.3	—	29.4 ± 3.2**	67.4 ± 5.3**	98.0 ± 6.5**	22.0
Dioscoreanone (**3**)	0.0 ± 4.7	22.3 ± 2.5*	56.6 ± 3.1**	88.7 ± 1.2**	105.8 ± 7.3**	8.1
Compound	% Inhibition at various concentrations (μM) against IL-4 release					IC_{50} (μM)
	0	3	10	30	100	
(B)						
Dioscorealide A (**1**)	0.0 ± 4.2	—	3.8 ± 3.8	38.5 ± 3.4**	90.2 ± 2.8**	36.2
Dioscorealide B (**2**)	0.0 ± 4.2	0.4 ± 1.4	0.6 ± 2.9	9.0 ± 2.5	72.4 ± 3.1**	73.6
Dioscoreanone (**3**)	0.0 ± 5.2	1.7 ± 4.4	93.2 ± 0.3**	94.6 ± 0.8**	99.5 ± 1.8**	6.0

Statistical significance, $^{*}p < 0.05$, $^{**}p < 0.01$.
[a] Each value represents means ± SEM of four determinations.

dioscoreanone (**3**) exhibited potent anti-cancer activity against MCF-7 and COR-L23 cell lines.[4,5]

In conclusion, the present study supports the use of *D. membranacea* for treatment of allergy and allergy-related diseases as claimed by Thai traditional doctors. Dioscorealide A (**1**), dioscorealide B (**2**), dioscoreanone (**3**), and diosgenin (**9**) are the active principles of this plant. These pure compounds may serve as starting points for the design of a range of novel semi-synthetic and synthetic compounds as anti-allergic medicinal agents in the future.

3. Experimental

3.1. Plant materials and preparation of extracts

Hua-Khao-Yen plants were collected from areas in southern, northern, and eastern provinces of Thailand. They were the rhizomes of *D. membranacea* Pierre ex Prain & Burkill (Amphur Pa-tue, Chumporn province), *D. birmanica* Prain & Burkill (Chantaburi Medicinal Plant Garden, Chuntaburee Province), *Smilax corbularia* Kunth (Amphur Mae-Tang, Chiengmai province), *S. glabra* Roxb. (Amphur Mueang, Loui province), and root of *Pygmaeopremna herbacea* Roxb. (Amphur Amnajcharoen, Ubonrajathanee province). These plants were identified by Assoc. Prof. Dr. Arunporn Itharat, and voucher specimens are deposited at the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.[5]

Plant materials were washed and then dried at 50 °C, powdered, and extracted by methods corresponding to those used by Thai traditional doctors. The water extracts were obtained by boiling dried plant material (100 g) for 30 min in 300 mL of distilled water, and the filtered extract was then freeze-dried. For the ethanolic extracts, the plant material (100 g) was percolated with 95% ethanol, then the filtered organic extract was concentrated to dryness under reduced pressure. The extracts were dissolved in dimethylsulfoxide (DMSO) before bioassay.

3.2. Isolation of compounds from *D. membranacea* extract

The rhizomes of *D. membranacea* (1 kg) were dried, powdered, and extracted with ethanol, and the ethanol concentrated under reduced pressure to obtain 31 g of

ethanolic extract. Crude ethanolic extract (10 g) was then chromatographed over silica gel using chloroform (10× 100 mL), chloroform/methanol (1:1, 10× 100 mL), and methanol (10× 100 mL), respectively. Each fraction was dried and evaporated to yield 2.8, 0.2, and 6.7 g, respectively, these fractions being noted as FA, FB and FC. The chloroform fraction (FA, 2 g), the most active fraction, was then re-chromatographed over silica gel using hexane, chloroform, and methanol gradient as follows; hexane/chloroform 6:4 (1000 mL), 8:2 (1000 mL), 95:5 (1000 mL), 9:1 (500 mL) and 7:3 (500 mL), respectively. Compound **1** (11.1 mg, 0.0048% w/w), **2** (31 mg, 0.0135% w/w), **3** (8.4 mg, 0.0036% w/w)[4], mixture of **4**[6] and **5**[7] (10 mg), **6**[8] (16.6 mg, 0.0072% w/w), **7**[9] (32.1 mg, 0.0140% w/w), and **8**[10] (15 mg, 0.0065% w/w) were afforded. Mixture of **4** and **5** was further separated by HPLC, RP-8 column using acetonitrile and water in the ratio of 86:4, at flow rate of 0.5 mL/min to obtain **4** (3.2 mg, 0.0013% w/w) and **5** (4.5 mg, 0.0019% w/w). Compound **9**, diosgenin, was obtained from the acid hydrolysis of **7**. The structures of all compounds were elucidated by chemical and spectroscopic means. The spectral data of each compound was compared with those of the reported ones.

3.3. Anti-allergic activity assay

3.3.1. Reagents. Minimum essential medium eagle (MEM) and anti-DNP-IgE (Monoclonal anti-DNP) were purchased from Sigma; fetal calf serum (FCS) was from Gibco; dinitrophenylated bovine serum albumin was prepared as described previously.[11] TNF-α and IL-4 test kits were from R&D Systems Inc. and Endogen, USA, respectively. Other chemicals were from Sigma. 24-well and 96-well plates were from Nunc.

3.3.2. Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 cells. Inhibitory effects on the release of β-hexosaminidase from RBL-2H3 [cell no CRL-2256 from American Type Culture Collection (ATCC)] were evaluated by the following modified method.[12] Briefly, RBL-2H3 cells were dispensed in 24-well plates at a concentration of 2×10^5 cells/well using MEM containing 10% FCS, penicillin (100 U/mL), streptomycin (100 U/mL), and anti-DNP IgE (0.45 μg/mL), then incubated overnight at 37 °C in 5% CO_2 for sensitization of the cells. The cells were washed twice with 500 μL of Siraganian buffer (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 0.1% bovine serum albumin (BSA), and 40 mM NaOH, pH 7.2) and then incubated in 160 μL of Siraganian buffer for an additional 10 min at 37 °C. After that, 20 μL of test sample solution was added to each well and incubated for 10 min, followed by addition of 20 μL of antigen (DNP-BSA, final concentration was 10 μg/mL) at 37 °C for 20 min to stimulate the cells to degranulate. The supernatant was transferred into 96-well plate and incubated with 50 μL of substrate (1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was stopped by adding 200 μL of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration was 0.1%). Under this condition, it was calculated that 50–60% of β-hexosaminidase was released from the cells in the control groups by determination of the total β-hexosaminidase activity after sonication of cell suspension. The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition } \% = [1 - (T - B - N)/(C - N)] \times 100$$

Control (C): DNP-BSA (+), Test sample (−); Test (T): DNP-BSA (+), Test sample (+); Blank (B): DNP-BSA (−), Test sample (+); Normal (N): DNP-BSA (−), Test sample (−).

3.3.3. β-Hexosaminidase inhibitory activity. In order to clarify that the anti-allergic effects of samples are due to the inhibition on hexosaminidase release, but not a false positive from inhibition of β-hexosaminidase activity, the following assay was then carried out.

The cell suspension (5×10^6 cells) in 10 mL of phosphate-buffered saline (PBS) was sonicated. The solution was then centrifuged; and the supernatant was diluted with Siraganian buffer and adjusted to equal the enzyme activity of the degranulation tested above. The enzyme solution (45 μL) and test sample solution (5 μL) were transferred into a 96-well microplate and incubated with 50 μL of the substrate solution at 37 °C for 1 h. The reaction was stopped by adding 200 μL of the stop solution. The absorbance was measured using a microplate reader at 405 nm.

3.3.4. Inhibitory effects on antigen-induced TNF-α and IL-4 release from RBL-2H3 cells. Inhibitory effects on the release of TNF-α and IL-4 from RBL-2H3 were evaluated by the method reported previously.[12] RBL-2H3 cells (2×10^5 cells/well) were sensitized with anti-DNP-IgE as described above. The cells were washed with MEM containing 10% FCS, penicillin (100 U/mL), and streptomycin (100 μg/mL), and exchanged with 320 μL of fresh medium. Then 40 μL of test sample solution and 40 μL of antigen (DNP-BSA, final concentration was 10 μg/mL) were added to each well and incubated at 37 °C for 4 h. The supernatant was transferred into 96-well ELISA plate and then TNF-α and IL-4 concentrations were determined using commercial ELISA kits. The test samples were dissolved in DMSO, and the solution was added to MEM (final DMSO was 0.1%). The inhibition on TNF-α and IL-4 production was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition } \% = [1 - (T - N)/(C - N)] \times 100$$

Control (C): DNP-BSA (+), Test sample (−); Test (T): DNP-BSA (+), Test sample (+); Normal (N): DNP-BSA (−), Test sample (−).

3.4. Statistics

The results were expressed as means ± SEM of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. Statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.

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