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Bioactive constituents from the leaves of *Clinacanthus nutans* Lindau

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ABSTRACT

Three chlorophyll derivatives (phaeophytins) were isolated from the chloroform extract of *Clinacanthus nutans* Lindau leaves by means of chromatographic techniques and bioactivity-guided fractionation to give three pure compounds. Structure elucidation of the isolated compounds was carried out on the basis of spectral analyses. Three of these were known compounds with structures related to chlorophyll a and chlorophyll b namely 13^2-hydroxy-(13^2-*R*)-phaeophytin b, 13^2-hydroxy-(13^2-*S*)-phaeophytin a and 13^2-hydroxy-(13^2-*R*)-phaeophytin a. These compounds, which have not previously been reported in this plant, were shown to have anti-herpes simplex activity. They exhibited anti-HSV-1F activity at subtoxic concentrations. Their inhibitory activity affected the virus before viral entry to the host cells. This effect might be virucidal or interference with viral adsorption or penetration.

1. Introduction

Herpes simplex virus (HSV) is highly inflectious and the prevalence of the antibodies to herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) in the normal populace was shown to be as high as 60% and 55%, respectively.[1] Acyclovir, the antiviral drug of choice for the treatment of HSV infection, is quite expensive and the consumption of this medicine in Thailand is increasing at approximately 20% annually.

Clinacanthus nutans (Burm. f.) Lindau (Thai name: Phaya Yo or Phaya Plong Thong) is a small shrub, native to tropical Asia, and often cultivated. *C. nutans* has long been used in Thailand as a traditional medicine for the treatment of skin rashes, insect and snakebite, herpes simplex virus (HSV), and varicella-zoster virus (VZV) lesions. Extracts from the leaves were reported to possess analgesic and anti-inflammatory activities,[2] antiviral activities against varicella-zoster virus[3] and herpes simplex virus type-2.[4] Clinical trials in patients with genital herpes are also reported.[5,6] However, negative results have also been reported.[7] Nonetheless clinical trials have reported the successful use of a *C. nutans* preparation (cream or lotion) for the relief of minor skin inflammation and insect bites, including treatment of genital herpes and varicella-zoster lesions in patients.[8]

C. nutans has been phytochemically and chemically investigated and previously stigmasterol,[9] lupeol, β-sitosterol,[10] belutin,[11] six known C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin-7-*O*-β-glucopyranoside, orientin, isoorientin,[12] five sulfur-containing glycosides,[13], two glycoglycerolipids,[14] a mixture of nine cerebrosides and a monoacylmonogalatosylglycerol,[15] have been isolated. Only the two glycoglycerolipids have been shown to exhibit antiviral activity.

The present communication reports a preliminary study initiated by the Medicinal Plant Research Institute, Department of Medical Science, Ministry of Public Health on antiviral compounds from *C. nutans* using bioassay-guided fractionation. The most antivirally active fractions were selected for further antiviral-guided fractionation by means of chromatographic techniques. This led to the isolation of three pure compounds, which were identified as chlorophyll a and chlorophyll b related compounds by spectroscopic methods, and the determination of their anti-HSV-1 activity.

2. Results and discussion

The structures of Compounds **1–3** were identified as chlorophyll a and chlorophyll b related compounds as follow (Fig. 1).

Compound **1** was obtained as a dark green amorphous solid. The IR, ^{1}H NMR and ^{13}C NMR data of **1** were found to be closely similar to those of compound **2**. By direct comparison of the ^{1}H

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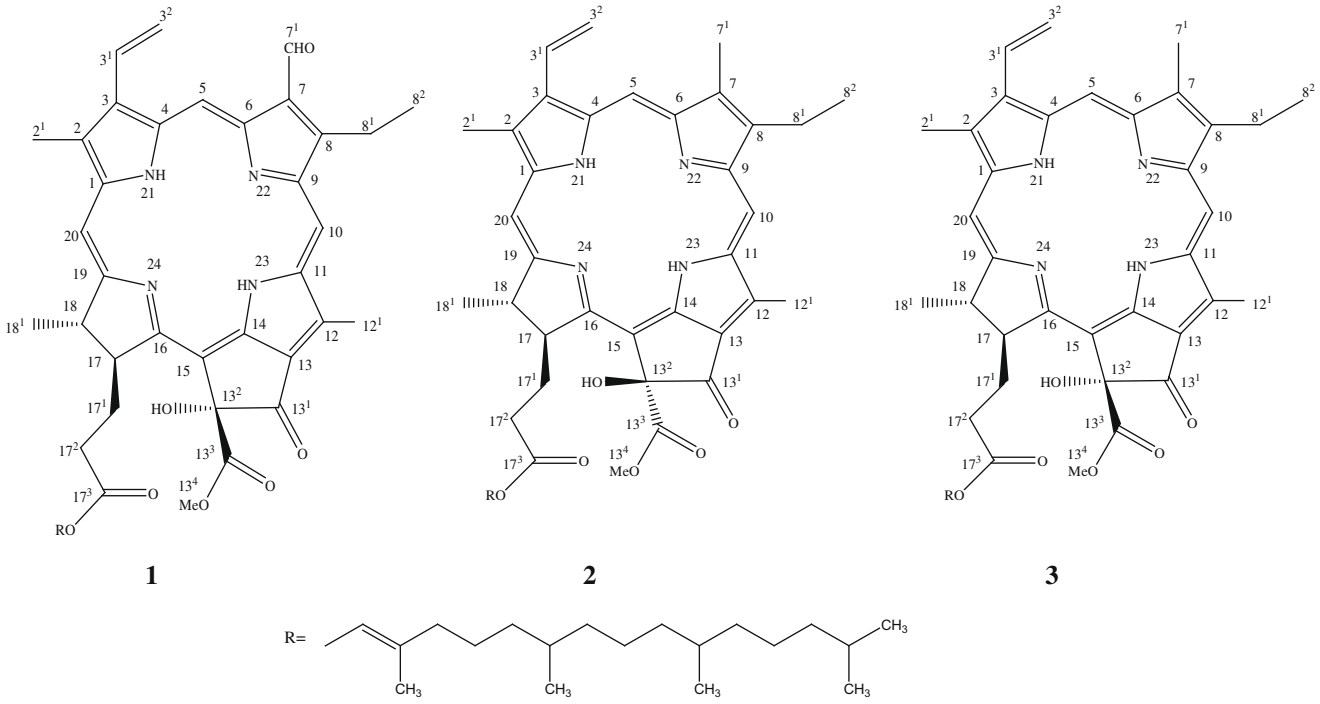

Figure 1. Structure of compounds **1**–**3**.

NMR and ^{13}C NMR data of compound **1** with those of the known compound 13^2-hydroxy-(13^2-*S*)-phaeophytin b[16,17] they were found to be closely equivalent indicating that compound **1** is 13^2-hydroxy-(13^2-*R*)-phaeophytin b (Fig. 1).

Compound **2** was obtained as a green powder. The IR spectrum display signal of amine, hydroxyl, and ester functional groups. The absolute configuration at C-13^2 of compound **2** was further confirmed by the observed correlations of H-17^1 to H-13^4 and H-17^2 to H-13^4 in the NOESY spectrum of compound **2**. These features indicated that the structure of **2** was similar to the known compound phaeophytin a.[18] Thus compound **2** was identified as 13^2-hydroxy-(13^2-*S*)-phaeophytin a (Fig. 1).

Compound **3** was obtained as a green powder. The IR, ^1H NMR and ^{13}C NMR data of **3** were found to be closely similar to those of compound **2.** By direct comparison of the ^1H NMR and ^{13}C NMR data of compound **3** with those of the known compound 13^2-hydroxy-(13^2-*R*)-phaeophytin a[18] they were closely equivalent indicating that compound **3** is 13^2-hydroxy-(13^2-*R*)-phaeophytin a (Fig. 1).

To study the anti-HSV-1F activity, all compounds were first subjected to a determination of cell cytotoxicity. Results showed that 5.89, 6.21 and 6.21 μM of compound **1, 2, 3**, respectively were the maximal concentration which were not toxic to Vero cells. Subtoxic concentrations of each compound were used in anti-HSV-1F study. DMSO with the same concentrations in diluted compound also did not affect the cell viability.

With respect to the anti-HSV-1F activity, subtoxic concentration of compounds **1**, **2** and **3** exhibited 100% inhibition activity as shown in Figure 2. IC50 of each compound was 1.96, 3.11 and 3.11 nM, respectively. When all three compounds were further evaluated for inhibition step of infection for the pre-viral entry and post-viral entry step, 100% inhibition activity of all compounds was demonstrated in pre-viral entry step (Fig. 2). This inhibitory effect on HSV-1F infectious dose at 1000 PFU/mL was time dependent. Plaque formation was completely inhibited at 30 min of virus-compounds incubation (data not shown). For post-viral entry, anti-HSV-1F activity showed about 30% of inhibition. These result suggested that these compounds affected on HSV-1F infection in the step before viral entry.

Compounds **1**–**3** might interfere with the virion envelope structures or a mask viral glycoproteins, which are necessary for adsorption and entry into host cells. The virus might also be directly inactivated by the compounds during incubation. The result is similar to the study by Schuhmacher et al. who showed that the virucidal effect of peppermint oil occurs on virus before the adsorption of HSV-1 and -2 to RC-37 cells.[19] Liu et al. showed anti-HSV-1 effects in pretreatment and treatment during virus infection with GLPG proteoglycan which was extracted and purified from the mycelia of *Ganoderma lucidum*. They suggested that GLPG inhibited viral replication by interfering with the early events of viral adsorption and entry into target cells.[20] This suggested that an interaction between chlorophyll a and chlorophyll b related compounds of *C. nutans* and HSV-1 has a different mechanism from ACV, which is the worldwide drug usage for HSV infection.

The three compounds inactivate HSV-1F before cell entry. The mechanisms may be binding of the compounds to viral glycoproteins involved in host cell adsorption and penetration, whereas the mechanism of ACV is interference with the viralDNA polymerase inside the infected cells.[21] Therefore, these compounds may be used in a synergistic treatment of HSV infection in the future.

3. Experimental

UV spectra were obtained with a Hewlett Packard 8452A diode array UV–vis spectrophotometer, IR spectra were measured with a Perkin–Elmer FT-IR 2000 spectrophotometer (KBr disk method), and Mass spectra were measured on a mass spectrometer (5989B Hewlet–Packard) FAB-MS; glycerol as matrix. The ^1H and ^{13}C NMR, DEPT, COSY, NOESY, HMQC, and HMBC spectra were recorded with a Bruker DRX 500 spectrometer in pyridine-d_5 solution and chemical shifts are expressed in δ (ppm) with reference

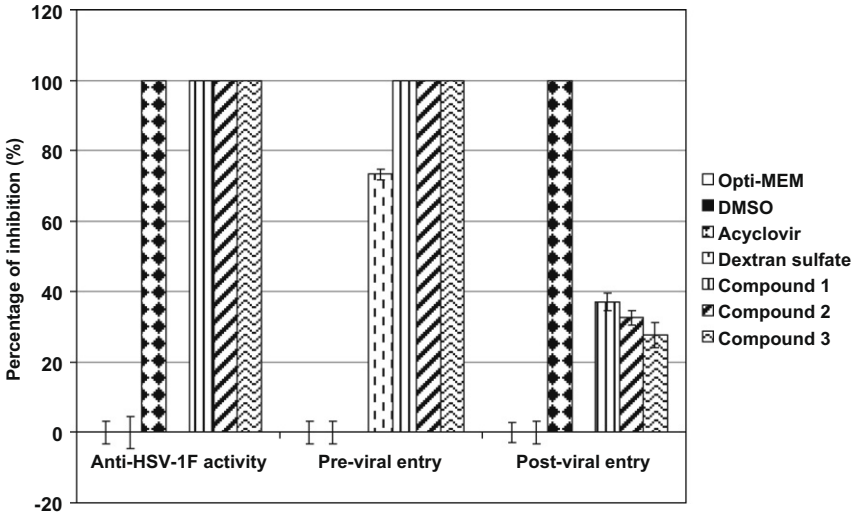

Figure 2. Mode of inhibitory activities of chlorophyll a and chlorophyll b related compounds against HSV-1F during different stages of the viral infection. Virus was treated with the maximal subtoxic concentration of the compounds, acyclovir or dextran sulfate was used as a control in each of experiment. Experiments were repeated independently two times and data presented are the mean of three experiments.

to the solvent signals. Silica gel 60 (70–230 mesh) and silica gel 60 PF 254 were used for column chromatography and preparative thin-layer chromatography, respectively. Solvents of technical grade were used for chromatographic purposes. Anisaldehyde-sulfuric acid spraying reagent (modification b) was prepared according to the method of Stahl (1965).

3.1. Plant material

Fresh aerial parts of C. *nutans* (Burm. f.) Lindau (Family Acanthaceae) were collected during October to December. The specimens were authenticated by the Botanical Section, Medicinal Plant Research Institute, Department of Medical Sciences, where voucher specimens (Bansiddhi 432) are deposited. The leaves were separated from the stems, washed thoroughly and dried in an oven at 50 °C. The dried sample was ground to powder.

3.2. Extraction and isolation

The dried powdered leaves (1.0 kg) were sequentially extracted with hexane and chloroform, respectively. The chloroform extract was concentrated in vacuo to give a residue (18.7 g) which was chromatographed on a silica gel 60 column. The column was eluted successively with hexane–ethyl acetate (1:1), ethyl acetate, chloroform–ethanol (1:1), and ethanol. Four major fractions (I, 6.65 g; II, 1.40 g; III, 6.24 g and IV, 0.97 g) were obtained by monitoring with TLC (toluene–petroleum ether–methanol–methyl ethyl ketone 30:60:5:5) All fractions were examined for anti-herpes simplex virus activity by the plaque reduction method. The most antivirally active fractions were selected for further purification. A portion of Fraction I (1.0017 g) was further separated by preparative thin-layer chromatography (hexane–ethyl acetate 7:3) to afford five fractions (A, 0.0450 g; B, 0.0406 g; C, 0.0791 g; D, 0.0697 g and E, 0.1469 g). Fraction C (0.0791 g) was further purified by preparative thin-layer chromatography using the same developing solvent to give crude compound **1** (0.0136 g), which was recrystallized from methanol (0.0065 g) Fraction D (0.0697 g) was further purified by preparative thin-layer chromatography using the same developing solvent to give crude compound **2** (0.0117 g) and compound **3** (0.0113 g), which were recrystallized from methanol (0.0061 g) and (0.0057 g), respectively.

3.3. 13^2-Hydroxy-(13^2-*R*)-phaeophytin b (1)

A dark green powder; FABMS *m/z* 901 $[M+H]^+$ (calcd for $C_{55}H_{72}N_4O_7$); UV/vis ($CHCl_3$) λ_{max} nm 412, 438, 520, 600 and 670; FT-IR (KBr) ν_{max} cm^{-1}: 3429, 2925, 2852, 1740, 1721, 1637 and 1300; ^1H NMR and ^{13}C NMR are the same as those published data.[16,17]

3.4. 13^2-Hydroxy-(13^2-*S*)-phaeophytin a (2)

A bright green color; FABMS *m/z* 887 $[M+H]^+$ (calcd for $C_{55}H_{74}N_4O_6$); UV/vis ($CHCl_3$) λ_{max} nm 408, 506, 536, 613 and 670; FT-IR (KBr) ν_{max} cm^{-1}: 3430, 2924, 1741, 1620 and 1460; ^1H NMR and ^{13}C NMR are the same as those published data.[18]

3.5. 13^2-Hydroxy-(13^2-*R*)-phaeophytin a (3)

A bright green color; FABMS *m/z* 887 $[M+H]^+$ (calcd for $C_{55}H_{74}N_4O_6$); UV/vis ($CHCl_3$) λ_{max} nm 412, 507, 537, 612 and 668; FT-IR (KBr) ν_{max} cm^{-1}: 3429, 2995, 1740, 1617 and 1455; ^1H NMR and ^{13}C NMR are the same as those published data.[18]

3.6. Anti-herpes simplex viral activity assay

The plaque reduction assay was employed using Vero cell line (African green monkey kidney cell line) and HSV-1 strain F (HSV-1F). Subtoxic concentrations of the compounds were determined before study of anti-HSV-1F activity. Briefly, Vero cells were seeded in a 96-well tissue culture plate at a density of 2×10^6 cells per well and incubated at 37 °C overnight. Then a cell monolayer was cultured in medium with or without serial twofold dilutions of each compound at 37 °C. After 72 h of treatment, cells were washed and stained with 3% crystal violet solution and dried at room temperature overnight. Stained crystal violet was dissolved in DMSO and OD_{620} of solution was measured. The percentage of cell viability was evaluated by comparing the OD value of sample to that of a cell control. Subtoxic concentration was the maximal concentration which had OD as the cell control and was used in anti-HSV-1F activity studies.

For the study of anti-HSV-1 activity, 1000 PFU/mL of HSV-1F were incubated with compounds at the subtoxic concentration or acyclovir (5 μg/mL) at 37 °C for 60 min. HSV-1F in cultured

medium and DMSO diluted to a concentration as in compound were used as viral and solvent control, respectively. After 60 min incubation, 50 μL of each mixture were adsorbed on confluent Vero cell at 37 °C for 1 h, subsequently the mixtures were aspirated. The infected cells and non-infected cells were cultured in medium with CMC containing compounds at 37 °C for 72 h. The viral plaques were counted and the percentage of inhibition was calculated as $(100\% \times (C - T)/C)$, where C and T refer to the plaque number in the absence and presence of the compound, respectively. Compounds which had % inhibitory activity more than 80 were further investigated for the step of inhibition: pre-viral entry or post-viral entry. Each of compounds was diluted in serial twofold dilution and used in the experiment for determination of IC50.

In pre-viral entry step, the subtoxic concentration of compounds and dextran sulfate (1 mg/mL) were incubated with 1000 PFU/mL of HSV-1F at 37 °C for various times (10, 30 and 60 min). HSV-1F in cultured medium and DMSO diluted to a concentration as in compound were used as viral and solvent control, respectively. After incubation, 50 μL of each mixture was adsorbed on confluent Vero cell and incubated for viral adsorbtion at 37 °C for 1 h. After virus adsorption, the mixtures were aspirated. The cells were washed and cultured in medium containing CMC at 37 °C for 72 h. The viral plaques were counted and the percentage of inhibitory activities of compounds and dextran sulfate on HSV-1F in pre-viral entry step were calculated.

In the post-viral entry step, the confluent Vero cells were adsorbed with 1000 PFU/mL of HSV-1F at 37 °C for 1 h. After viral adsorption, the excess viruses were aspirated and the cells were washed and cultured in medium with CMC containing subtoxic concentration of compounds. The viral controls were cultured in cultured medium with CMC and acyclovir was used as drug control. After incubation at 37 °C for 72 h, the viral plaques were counted and the percentage of inhibitory activities of compounds were calculated.

In conclusion, we have discovered the inhibitory activities against HSV-1F in pre-viral entry step but not in post-viral entry step of chlorophyll a and chlorophyll b related compounds from *C. nutans* an important Thai medicinal plant used for herpes infections in primary health care. These compounds are shown to have anti-herpes simplex activity for the first time in this plant. By using suitable analytical methods, these compounds will be further used as markers for qualitative control of the preparations made from the plant extract. Further studie on using these compounds as markers are being conducted at the Department of Medical Science Ministry of Public Health.

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