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ORIGINAL ARTICLE

Geraniinic acid derivative from the leaves of *Phyllanthus reticulatus*

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Abstract

Chemical constituents as well as cytotoxic and insecticidal activity of the crude methanol extract from the leaves of *Phyllanthus reticulatus* Poir. (Euphorbiaceae) were investigated. (5*R**,6*R**)-4,6-Dimethoxycarbonyl-5-[2′,3′,4′-trihydroxy-6′-(methoxycarbonyl) phenyl]-5,6-dihydro-2*H*-pyran-2-one (**1**) along with 3,4,3′-tri-*O*-methylellagic acid, and methyl gallate were isolated from the dichloromethane extract. Determination of their structures was based on spectroscopic analysis. Compound **1** possessed a very weak insecticidal activity against *Spodoptera frugiperda* (Sf9) with an IC_{50} value of 27.27 µg/mL.

Keywords: Euphorbiaceae; geraniinic acid; insecticidal activity; *Phyllanthus reticulatus*; structure elucidation

Introduction

Phyllanthus reticulatus Poir. (Euphorbiaceae) is a shrub easily grown and distributed widely in Thailand. It has been used as a folklore medicine in Thailand for the treatment of asthma, anemia, fever, and thirst, or used as a diuretic (Pangthong et al., 1986), astringent, and anti-inflammatory agent (Poopatanapong & Wongprasert, 1987). An infusion of dried leaves has been used as a diuretic and external astringent for sores, burns, suppuration, chafes, and venereal sores in human adults in East Africa (Hedberg et al., 1983), Tanzania (Chhabra et al., 1984), and Malaysia (Ilham et al., 1995). In India, the leaf juice has been used as human adult antivenin (Selvanyagam et al., 1994) and for diarrhea in children (Jayaweera, 1980). Previously, there have been some reports on antibacterial (Sawhney et al., 1978; Khan et al., 1980) and antimalarial (Omulokoli et al., 1997) activity from the leaf extract of this plant. Compounds reported earlier from various parts of *P. reticulatus* were such as pyrogallic acid, ellagic acid, *p*-coumaric acid (Neves & Neves, 1966), betulinic acid, glochidonol (Hui et al., 1976), *epi*-friedelanol (Chandler & Hooper, 1979), taraxerone (Joshi et al., 1981), 3,3′,4-tri-*O*-methylellagic acid, pirorisinol (Sangkasila, 1998), and tricin (Jain & Nagpal, 2002). This article presents the isolation and structure elucidation of chemical constituents from the leaves of *P. reticulatus* as well as their cytotoxic and insecticidal activity.

Materials and methods

General experimental procedures

^{1}H-Nuclear magnetic resonance (NMR) (300 MHz) and ^{13}C-NMR (75.4 MHz) experiments were performed on an Ultrashield™ 300 spectrometer, Bruker®. Chemical shifts were recorded as parts per million (ppm) on the δ scale, using tetramethylsilane (TMS) as internal standard (Scientific and Technological Research Equipment Center, Silpakorn University).

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Plant collection and authentication

P. reticulatus leaves were collected from Nakhon Pathom Province, Thailand in September 2004. Authentication was performed by comparison with a herbarium specimen (collection no. BKF 127336) at the Forest Herbarium, Royal Forest Department, Bangkok, Thailand. A voucher specimen has been deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Silpakorn University, Thailand.

Extraction and isolation

The dried ground leaves of *P. reticulatus* (2.2 kg) were macerated with methanol (10 L) three times to furnish the crude methanol extract (610.4 g). The crude methanol extract was mixed with kieselguhr (1.8 kg), and continuous extraction was performed to yield hexane (68.2 g), dichloromethane (12.9 g), and methanol (41.8 g) extracts, respectively. The dichloromethane extract was subjected to column chromatography using Sephadex LH-20 and a mixture of CH_2Cl_2:MeOH (1:1) as a mobile phase to yield five main fractions (C1–C5). The second fraction (C2, 3.4856 g) was submitted to Sephadex LH-20 column chromatography, employing a gradient system of 50% hexane–CH_2Cl_2 to 50% CH_2Cl_2–acetone as eluent, to give three fractions (C21, C22, C23). The second fraction (C22) was further purified on a silica gel column using the gradient system of 50% hexane–CH_2Cl_2 to 50% CH_2Cl_2–acetone, and compound **1** (64.0 mg) was crystallized from the saturated acetone solution by adding hexane and allowing it to stand for 24 h in a cool place. Fraction C21 (0.6 g) was chromatographed on a silica gel column and eluted with 3–100% EtOAc in $CHCl_3$ to yield 3,4,3′-tri-*O*-methylellagic acid (4.9 mg). Successive column chromatography of fraction C4 from the dichloromethane extract on Sephadex LH-20 with a mixture of hexane and acetone (50:50) yielded methyl-3,4,5-trihydroxybenzoate (methyl gallate) (81.8 mg).

(5*R,6*R**)-4,6-Dimethoxycarbonyl-5-[2′,3′,4′-trihydroxy-6′-(methoxycarbonyl) phenyl]-5,6-dihydro-2*H*-pyran-2-one (1)**

Colorless needles, $[\alpha]_D^{29.1}$ =+171.5° (*c* 0.52, Me_2CO). EIMS (70 eV): *m/z* 396 $[M]^+$ (33.6), 364 (30.3), 332 (75.2), 305 (100), 273 (75.2), 245 (56.3), 217 (21.8). ^{1}H-NMR (acetone-d_6, 300 MHz): 7.14 (1H, *s*, H-5′), 6.82 (1H, *d*, *J*=0.9 Hz, H-3), 5.41 (1H, *dd*, *J*=0.9, 1.8 Hz, H-5), 5.28 (1H, *d*, *J*=1.8 Hz, H-6), 3.68 (3H, *s*, $COOCH_3$-6), 3.64 (3H, *s*, $COOCH_3$-4), 3.63 (3H, *s*, $COOCH_3$-6′). ^{13}C-NMR (acetone-d_6, 75 MHz) (Table 1).

3,4,3′-tri-*O*-Methylellagic acid

Colorless needles. EIMS (70 eV): *m/z* 344 $[M]^+$ (66.4), 329 (13.4), 286 (12.6), 273 (5.04), 258 (4.2), 241 (4.2), 149 (8.4), 129 (9.2). ^{1}H-NMR ($CDCl_3$, 300 MHz): 7.77 (1H, *s*), 7.68 (1H, *s*), 6.26 (1H, *s*, OH), 4.41 (3H, *s*, OCH_3), 4.24 (3H, *s*, OCH_3), 4.04 (3H, *s*, OCH_3). ^{13}C-NMR ($CDCl_3$, 75 MHz): 111.58, 107.76, 62.17 (OCH_3), 61.99 (OCH_3), 56.84 (OCH_3).

Methyl-3,4,5-trihydroxybenzoate (methyl gallate)

Pale yellow amorphous solid. EIMS (70 eV): *m/z* 185 $[M + 1]^+$ (21.8), 184 $[M^+]$ (49.6), 153 (100.0), 125 (21.0). ^{1}H-NMR (acetone-d_6, 300 MHz): 7.12 (2H, *s*, H-2,4), 3.79 (3H, *s*, OCH_3-7). ^{13}C-NMR (acetone-d_6, 75 MHz):

Table 1. NMR chemical shifts (ppm) of geraniinic acid (Foo, 1995) and compound **1** in acetone-d_6.

	Geraniinic acid			Compound **1**		
Carbon no.	C	H	HMBC correlation	C	H	HMBC correlation
2	162.8			162.90		
3	122.6	6.44	37.8, 107.9, 116.3, 144.7, 162.8, 164.9	128.89	6.82	34.34, 141.85, 166.02
4	144.7			141.85		
5	37.8	5.35	80.5, 116.3, 122.6, 144.8, 172.0	34.34	5.41	78.27, 115.01, 117.53, 141.85, 142.69, 128.89, 166.02, 169.56
6	80.5	5.11	37.8, 116.3, 144.8, 145.5, 162.8, 172.0	78.27	5.28	34.34, 115.01, 141.85, 162.90, 169.56
1′	116.3			115.01		
2′	144.8			142.69		
3′	136.3			137.98		
4′	145.5			145.06		
5′	107.9	6.70	115.2, 136.3, 145.5, 168.9	107.73	7.14	115.01, 117.53, 137.98, 145.06
6′	115.2			117.53		
$\underline{C}OOCH_3$-4	164.9			166.02		
$\underline{C}OOCH_3$-6	172.0			169.56		
$\underline{C}OOCH_3$-6′	168.9			165.17		
$COO\underline{C}H_3$-4	—			51.95	3.64	166.02
$COO\underline{C}H_3$-6	—			52.27	3.68	169.56
$COO\underline{C}H_3$-6′	—			51.51	3.63	165.17

166.37 (C-7), 145.17 (C-3,5), 137.85 (C-4), 120.84 (C-1), 108.89 (C-2,6), 51.04 (OCH_3-7).

Cytotoxicity assay

The cytotoxicity assay was performed by the National Center for Genetic Engineering and Biotechnology (BIOTEC, Thailand), using human tumor cell lines: small cell lung cancer (NCI-H187), mouth carcinoma (KB), and breast cancer (MCF7). The tumor cell lines were plated overnight in 96-well microplates. Serial dilutions of the test samples were added and cells were incubated for 4–6 days. Cell growth was measured by colorimetric methods using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983) and sulforhodamine B (SRB) assay (Rubinstein et al., 1990). Positive controls were doxorubicin (IC_{50} of 33.7×10^{-2} µg/mL, 17.3×10^{-2} µg/mL, and 8.3×10^{-2} µg/mL for NCI-H187, KB, and MCF7, respectively) and ellipticine (IC_{50} of 33.2×10^{-2} µg/mL, 30.3×10^{-2} µg/mL, and 5.2×10^{-2} µg/mL for NCI-H187, KB, and MCF7, respectively).

Insecticidal assay

Compound **1** was tested for insecticidal activity against *Spodoptera frugiperda* (Sf9) by a colorimetric method, using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay with some modification (Salehzadeh et al., 2002; Fornelli et al., 2004). The Sf9 cells were cultured at 27°C in Grace's insect medium supplemented with 10% fetal calf serum (FCS) (v/v); 5×10^4 cells/well (100 µL) were plated in 96-well tissue culture plates and incubated at 27°C for 24 h. Dilutions of compound **1** ranging from 0.005 to 5 g/well were added into the wells and incubated at 27°C for 24 h. The wells were washed with phosphate buffered saline (PBS). New Grace's insect medium (100 µL) was added, co-cultured with 10 µL of 5 mg/mL MTT reagent in the dark, and incubated at 27°C for 3 h until a purple precipitate was visible. The medium was discarded and washed with PBS twice. Dimethylsulfoxide (DMSO) (100 µL) was added and left at room temperature in the dark for 20 min. This was then shaken by tapping and absorbance determined at 550 nm using a Fusion Universal Microplate Analyzer (Packard Bioscience Company). The 50% inhibiting concentration (IC_{50}) was calculated from dose–response curves to evaluate the cytotoxicity of compound **1** against Sf9. The assay was performed in triplicate, including blank wells containing medium only and untreated control cells with the presence of 0.5% DMSO instead of compound **1**. The positive control was rotenone at IC_{50} = 0.1 µg/mL.

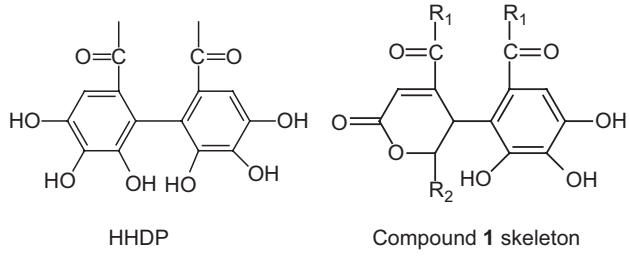

Figure 1. Hexahydroxydiphenoyl (HHDP) moiety (left) and compound **1** skeleton (right).

Results and discussion

The hexane and methanol extracts of *P. reticulatus* leaves were inactive in the *in vitro* cytotoxicity study. The dichloromethane extract showed IC_{50} values of 11.89 µg/mL in KB and 16.08 µg/mL in MCF7, but was inactive in the NCI-H187 human tumor cell line. The dichloromethane extract was then further purified using column chromatography, resulting in the separation of compound **1** and other known compounds. Compound **1** was isolated as colorless needles. From the ^{13}C-NMR spectrum (Table 1), there were 17 carbons, consisting of four carbonyl carbons (169.56, 166.02, 165.17, 162.90), eight aromatic carbons (145.06, 142.69, 141.85, 137.98, 128.89, 117.53, 115.01, 107.73), three methoxyl carbons (52.27, 51.95, 51.51), an oxygenated methine carbon (78.27), and an aliphatic methine carbon (34.34). The ^{1}H-NMR spectrum showed the presence of two aromatic protons (7.14, 6.82), two methine protons (5.41, 5.28), and three methoxyl protons (3.68, 3.64, 3.63). In electron impact mass spectrometry (EIMS), the molecular ion at *m/z* 396 suggested the possible molecular formula of $C_{17}H_{16}O_{11}$. Heteronuclear multiple quantum coherence (HMQC) spectra indicated four methine groups, the proton (5.28) associated with the oxygenated carbon (78.27), and the proton (5.41) associated with the aliphatic carbon (34.34). The deshielded proton at 6.82 was attributed to olefinic carbon (128.89) and the sharp singlet signal at 7.14 ppm was bonded to aromatic carbon (107.73). A small vicinal coupling (J = 1.8 Hz) between these two methine protons indicated a *trans* relationship with respect to their relative orientation. Moreover, the splitting pattern of doublets of doublets at 5.41 of H-5 with J = 0.9 Hz indicated a *meta* coupling with the olefinic proton (H-3, 6.82) which was attributed to the "W conformation" of the four bonds between them. These spectral data were consistent with a cyclic lactone structure linked to gallate as shown in Figure 1. This cyclohexenone portion was derived from the hexahydroxydiphenoyl (HHDP) moiety, which is a characteristic structural element found in the biosynthesis of tannins (Khanbabaee & Ree, 2001).

Heteronuclear multiple bond coherence (HMBC) showed a correlation between the protons of methoxyl

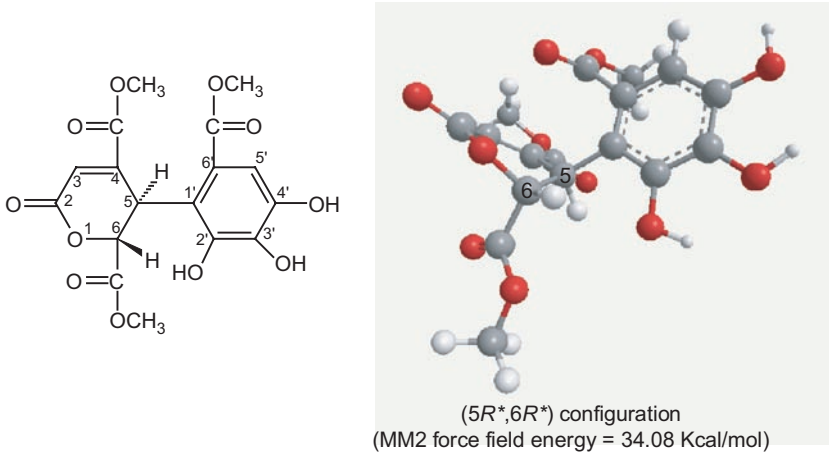

Figure 2. The structure of (5*R**,6*R**)-4,6-dimethoxycarbonyl-5-[2′,3′,4′-trihydroxy-6′-(methoxycarbonyl) phenyl]-5,6-dihydro-2*H*-pyran-2-one (compound **1**).

ester groups and carbonyl carbons that attached to C-4, C-6, and C-6′, respectively. Compound **1** consisted of two chiral carbons at C-5 and C-6. The small vicinal coupling (J = 1.8 Hz) between them indicated the *trans* relative configuration as found in positions 5 and 6 of geraniinic acid, reported by Foo (1995) recently. Consequently, the structure of compound **1** was unambiguously confirmed as (5*R**,6*R**)-4,6-dimethoxycarbonyl-5-[2′,3′,4′-trihydroxy-6′-(methoxycarbonyl) phenyl]-5,6-dihydro- 2*H*-pyran-2-one (Figure 2), a methylester subunit of geraniinic acid. The comparison of the NMR data between geraniinic acid and compound **1** is shown in Table 1.

Two other compounds isolated from the dichloromethane extract were identified as 3,4,3′-tri-*O*-methylellagic acid, and methyl-3,4,5-trihydroxybenzoate (methyl gallate) by the spectrometric methods.

Compound **1** was inactive (IC_{50} > 20 µg/mL) in the human tumor cell line cytotoxic assays (NCI-H187, KB, and MCF7). However, this compound exhibited a very weak insecticidal activity against *Spodoptera frugiperda* (Sf9) with an IC_{50} of 27.27 µg/mL, compared with rotenone (IC_{50} = 0.1 µg/mL).

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Declaration of interest

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