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SHORT COMMUNICATION

Cytotoxic constituents from the seeds of Vietnamese *Caesalpinia sappan*

Manh Hung Tran[1], Mai Thi Thanh Nguyen[1], Hai Dang Nguyen[2], Tien Dat Nguyen[2], and Thien Thuong Phuong[3]

[1]*Faculty of Chemistry, University of Science, Vietnam National University-HoChiMinh City, HoChiMinh City, Vietnam,* [2]*Institute of Marine Biochemistry, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam, and* [3]*Department of Pharmaceutical Analysis and Standardization, Vietnam National Institute of Medicinal Materials (VNIMM), Hanoi, Vietnam*

Abstract

Context: *Caesalpinia sappan* Linn. (Leguminosae) has been used in folk medicines for the treatment of many diseases. The heartwood of this plant contains various phenolic components with interesting biological applications; however, the chemical and biological potentials of the seed of this plant have not been fully explored.
Objective: This study identified the cytotoxic activity of compounds from the seeds of *C. sappan*.
Materials and methods: The methanol extract of the seed of *C. sappan* was suspended in H_2O and then partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH, successively. Diterpenoid compounds were isolated from the CH_2Cl_2-soluble fraction by silica gel column chromatography methods using organic solvents. The compound structures were determined by detailed analysis of NMR and MS spectral data. Cytotoxic activity was measured using a modified MTT assay against HL-60, HeLa, MCF-7, and LLC cancer cells. The activation of caspase-3 enzyme and western blotting assay were performed to confirm inhibitory mechanism of active compound.
Results: Five cassane-type diterpenoids were isolated and identified as phanginin I (**1**), phaginin A (**2**), phanginin D (**3**), phanginin H (**4**), and phanginin J (**5**). Compounds **1**–**4** showed effective inhibition against HL-60 cells with the IC_{50} values of 16.4 ± 1.5, 19.2 ± 2.0, 11.7 ± 1.6, and 22.5 ± 5.1 μM. Compounds **1**–**3** exhibited cytotoxic activity against HeLa cells with the IC_{50} values of 28.1 ± 3.6, 37.2 ± 3.4, and 22.7 ± 2.8 μM. Treatment of HL-60 cell lines with various concentrations of **3** (0–30 μM) resulted in the growth inhibition and induction of apoptosis.
Conclusion: These findings demonstrate that compound **3** (phanginin D) is one of the main active components of the seed of *C. sappan* activating caspases-3 which contribute to apoptotic cell death.

Keywords

Cassane-type diterpene, cytotoxicity, human promyelocytic leukemia, leguminosae

History

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Introduction

Caesalpinia sappan Linn. (Leguminosae) is distributed in Southeast Asia. It is used as an herbal medicine for the treatment of inflammation and to improve blood circulation (Nagai et al., 1984), influenza, allergic, and neuroprotective activities (Liu et al., 2009; Shen et al., 2007; Yodsaoue et al., 2009). This plant was used in Vietnamese traditional medicine for the treatment of menstrual and post-partum hematometra, trauma blood static, dizziness, post-partum blood losses, bloody dysentery, enteralgia, intestinal hemorrhage, infectious diarrhea, and for washing wounds (Bich, 1999). Many reports showed that the main components in the heartwood of *C. sappan* are phenolics with anti-inflammatory, antibacterial, and anti-influenza activities (Choi et al., 2007; Cuong et al., 2012; Kim et al., 2012; Namikoshi et al., 1987; Shimokawa et al., 1985; Washiyama et al., 2009). Nguyen et al. (2004) reported that the methanolic extract of the heartwood of *C. sappan* collected in Vietnam exhibited significant XO inhibitory activity. However, limited information is available concerning the principles of the seeds of *C. sappan*. The first chemical study on the seeds of *C. sappan* collected in Thailand led to the isolation of a serial of cassane-type diterpenes with cytotoxic effect against KB cell line (Yodsaoue et al., 2008). Phytochemical investigation on the seeds collected in China led to the isolation of several cassane diterpenoids with moderate cytotoxicity against HepG2 cell line (Zhang et al., 2012). Cleistanthane diterpene skeleton was also isolated from the seed of this plant and tested for antiausterity activity against human pancreatic cancer cell (Nguyen et al., 2013). In our experiment, further study on the phytochemical and biological activities from the seed of *C. sappan* collected in Vietnam resulted in the isolation of five diterpenes (**1**–**5**). This study evaluates the bioactive substances responsible for *in vitro* cytotoxic and apoptosis induction on HL-60, human premyelocytic leukemia cancer cell lines.

Correspondence: Manh Hung Tran, PhD, Faculty of Chemistry, University of Science, Vietnam National University-HoChiMinh City, 227 Nguyen Van Cu, District 5, HoChiMinh City, Vietnam. Tel: +84 8 3832 4457. Fax: +84 8 3835 0096. E-mail: tmhung801018@gmail.com

Materials and methods

General experimental procedures

Optical rotation was measured with a JASCO DIP 370 digital polarimeter (JASCO, Tokyo, Japan). UV spectra were

obtained in MeOH using a Thermo 9423AQA2200E UV spectrometer (JEOL, Tokyo, Japan), and IR spectra were obtained on a Bruker Equinox 55 FT-IR spectrometer (Bruker, Rheinstetten, Germany). The nuclear magnetic resonance (NMR) spectra were obtained on Varian Unity Inova 400 MHz spectrometer (Agilent Technologies, Inc., Santa Clara, CA). Silica gel (Merck, Darmstadt, Germany, 63–200 μm particle size) and RP-18 silica gel (Merck, Darmstadt, Germany, 75 μm particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F_{254} and RP-18 F_{254} plates (Merck, Darmstadt, Germany). HPLC was performed using a Waters 600 Controller system (Waters, Milford, MA) with a UV detector and an YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size, YMC Co., Ltd., Kyoto, Japan) and HPLC solvents were obtained from Burdick & Jackson, Muskegon, MI.

Plant materials

The seeds of *C. sappan* L. were collected in An Giang province of Vietnam, in September 2010. Professor Tran Cong Luan at Vietnam National Institute of Medicinal Material botanically authenticated the plant, where a voucher specimen number TCL-00120-A describing the plant is deposited.

Extraction and isolation

The air dried and powered seeds of *C. sappan* (200 g) were extracted three times (2 h × 1 L) with refluxing methanol. After the solvent was removed under reduced pressure, the residue was suspended in distilled H_2O and then partitioned with CH_2Cl_2, EtOAc, and *n*-BuOH, successively. The CH_2Cl_2-soluble fraction (4.2 g) was chromatographed on a silica gel column using a stepwise gradient of *n*-hexane:acetone (50:1–0:1) to yield 10 sub-fractions (CH.1–CH.10) according to their TLC profiles. Fraction CH.2 (0.55 g) was subjected to silicagel column chromatography and eluted with hexane:EtOAc (from 10:1) to afford compound **1** (14 mg). Fraction C.3 (0.81 g) was recrystallized from CH_2Cl_2 to yield compound **2** (61 mg). Further purification of CH.6 (0.85 g) by silicagel column chromatography using CH_2Cl_2:acetone (20: and 10:1) resulted in the isolation of compounds **3** (11.7 mg) and **4** (9 mg). Further purification of CH.8 (146 mg) by semi-preparative Waters HPLC systems [using an isocratic solvent system of 35% MeOH in H_2O + 0.1% trifluoroacetic acid (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of compound **5** (5.7 mg).

Identification

Phanginin I (**1**): White amorphous solid; [α]25D + 48.2 (*c* 0.01, $CHCl_3$); IR ν_{max} (KBr): 2959, 1725, 1126, 765 cm^{-1}; UV (MeOH) λ_{max} (log ε): 220 (3.80) nm; ^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm): 0.95 (1H, m, H-1a), 2.50 (1H, brd, *J* = 12 Hz, H-1b), 1.61 (1H, m, H-2a), 1.79 (1H, m, H-2b), 1.55 (1H, m, H-3a), 1.78 (1H, m, H-3b), 2.26 (1H, dd, *J* = 12.0, 2.0 Hz, H-5), 1.42 (1H, m, H-6a), 2.20 (1H, m, H-6b), 1.70 (1H, m, H-7a), 2.05 (1H, m, H-7b), 2.06 (1H, m, H-8), 1.85 (1H, m, H-9), 2.70 (1H, dd, *J* = 17.0, 7.0 Hz, H-11a), 2.10 (1H, m, H-11b), 2.68 (1H, q, *J* = 7.2 Hz, H-14), 6.20 (1H, d, *J* = 1.8 Hz, H-15), 7.22 (1H, d, *J* = 1.8 Hz, H-16), 0.98 (3H, d, *J* = 7.2 Hz, H-17), 1.05 (3H, s, H-19), 9.62 (1H, s, H-20), 3.68 (3H, s, 18-OMe); ^{13}C NMR (100 MHz, CD_3OD) δ_C (ppm): 31.5 (C-1), 18.6 (C-2), 35.7 (C-3), 50.5 (C-4), 49.7 (C-5), 23.2 (C-6), 31.0 (C-7), 35.9 (C-8), 44.5 (C-9), 48.2 (C-10), 22.1 (C-11), 148.5 (C-12), 123.4 (C-13), 32.1 (C-14), 110.3 (C-15), 141.3 (C-16), 17.0 (C-17), 179.5 (C-18), 15.2 (C-19), 209.7 (C-20), 53.5 (OMe).

Phaginin A (**2**): White solid; [α]25D –32.5 (*c* 0.01, $CHCl_3$); IR ν_{max} (KBr): 3440, 1725, 1102, 780 cm^{-1}; UV (MeOH) λ_{max} (log ε): 218 (3.86) nm; ^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm): 1.17 (1H, m, H-1a), 2.02 (1H, m, H-1b), 1.60 (1H, m, H-2a), 1.62 (1H, m, H-2b), 1.85 (1H, m, H-3a), 1.89 (1H, m, H-3b), 1.62 (1H, m, H-5), 1.15 (1H, m, H-6a), 2.24 (1H, m, H-6b), 1.42 (1H, m, H-7a), 1.68 (1H, m, H-7b), 2.52 (1H, m, H-8), 1.45 (1H, m, H-9), 2.63 (1H, dd, *J* = 16.0, 11.5 Hz, H-11a), 2.75 (1H, dd, *J* = 16.0, 6.0 Hz, H-11b), 2.50 (1H, m, H-14), 6.15 (1H, d, *J* = 1.8 Hz, H-15), 7.20 (1H, d, *J* = 1.8 Hz, H-16), 0.95 (3H, d, *J* = 7.0 Hz, H-17), 4.50 (1H, d, *J* = 11.5 Hz, H-19a), 3.65 (1H, d, *J* = 11.5 Hz, H-19b), 5.15 (1H, s, H-20), 3.72 (3H, s, OMe); ^{13}C NMR (100 MHz, CD_3OD) δ_C (ppm): 37.5 (C-1), 19.4 (C-2), 35.7 (C-3), 45.8 (C-4), 45.2 (C-5), 22.9 (C-6), 30.5 (C-7), 36.0 (C-8), 42.5 (C-9), 38.5 (C-10), 22.2 (C-11), 149.5 (C-12), 122.7 (C-13), 32.7 (C-14), 109.4 (C-15), 140.5 (C-16), 17.1 (C-17), 174.8 (C-18), 60.5 (C-19), 96.5 (C-20), 51.5 (OMe).

Phanginin D (**3**): White solid; [α]25D + 36.7 (*c* 0.01, $CHCl_3$); IR ν_{max} (KBr): 3442, 1730, 756 cm^{-1}; UV (MeOH) λ_{max} (log ε): 216 (4.05) nm; ^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm): 1.18 (1H, m, H-1a), 1.95 (1H, m, H-1b), 1.62 (1H, m, H-2a), 1.65 (1H, m, H-2b), 1.62 (1H, m, H-3a), 1.95 (1H, m, H-3b), 1.70 (1H, m, H-5), 1.12 (1H, m, H-6a), 2.15 (1H, m, H-6b), 1.32 (1H, m, H-7a), 1.67 (1H, m, H-7b), 2.45 (1H, m, H-8), 1.52 (1H, m, H-9), 2.40 (1H, m, H-11a), 2.72 (1H, dd, *J* = 16.0, 6.0 Hz, H-11b), 2.60 (1H, m, H-14), 6.15 (1H, d, *J* = 1.8 Hz, H-15), 7.22 (1H, d, *J* = 1.8 Hz, H-16), 0.96 (3H, d, *J* = 7.0 Hz, H-17), 4.85 (1H, s, H-19), 4.02 (1H, brd, *J* = 11.0 Hz, H-20a), 3.54 (1H, d, *J* = 11.0 Hz, H-20b), 3.70 (3H, s, 18-OMe), 3.42 (3H, s, 19-OMe); ^{13}C NMR (100 MHz, CD_3OD) δ_C (ppm): 38.2 (C-1), 20.4 (C-2), 37.1 (C-3), 46.8 (C-4), 44.2 (C-5), 22.7 (C-6), 30.4 (C-7), 37.0 (C-8), 42.2 (C-9), 37.1 (C-10), 22.4 (C-11), 149.0 (C-12), 122.3 (C-13), 31.4 (C-14), 109.5 (C-15), 140.5 (C-16), 17.3 (C-17), 174.9 (C-18), 101.5 (C-19), 63.5 (C-20), 51.5 (18-OMe), 54.6 (19-OMe).

Phanginin H (**4**): White solid; [α]25D + 18.2 (*c* 0.01, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 216 (3.86) nm; ^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm): 1.20 (1H, m, H-1a), 2.02 (1H, m, H-1b), 1.62 (1H, m, H-2a), 1.65 (1H, m, H-2b), 1.60 (1H, m, H-3a), 1.85 (1H, m, H-3b), 2.12 (1H, d, *J* = 11.6 Hz, H-5), 1.15 (1H, m, H-6a), 1.45 (1H, m, H-6b), 1.46 (1H, m, H-7a), 1.75 (1H, m, H-7b), 2.05 (1H, m, H-8), 1.85 (1H, dd, *J* = 12.0, 4.0 Hz, H-9), 4.85 (1H, d, *J* = 4.0 Hz, H-11), 2.65 (1H, m, H-14), 6.20 (1H, d, *J* = 1.8 Hz, H-15), 7.30 (1H, d, *J* = 1.8 Hz, H-16), 0.97 (3H, d, *J* = 7.0 Hz, H-17), 0.95 (3H, s, H-19), 3.95 (1H, brdd, *J* = 8.0 Hz, H-20a), 3.92 (1H, d, *J* = 8.0 Hz, H-20b), 3.70 (3H, s, 18-OMe); ^{13}C NMR (100 MHz, CD_3OD) δ_C (ppm): 36.2 (C-1), 17.6 (C-2), 37.0 (C-3), 46.9 (C-4), 46.2 (C-5), 25.0 (C-6), 30.5 (C-7), 37.3 (C-8), 49.2 (C-9), 46.0 (C-10), 69.3 (C-11), 148.0 (C-12), 125.3 (C-13),

31.6 (C-14), 109.2 (C-15), 142.5 (C-16), 15.3 (C-17), 178.9 (C-18), 14.6 (C-19), 70.5 (C-20), 52.5 (18-OMe).

Phanginin J (**5**): White solid; [α]25D + 27.5 (*c* 0.01, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 218 (3.98) nm; ^{1}H NMR (400 MHz, CD_3OD) δ_H (ppm): 0.92 (1H, m, H-1a), 2.45 (1H, brd, J = 12 Hz, H-1b), 1.69 (1H, m, H-2a), 1.82 (1H, m, H-2b), 1.65 (1H, m, H-3a), 2.15 (1H, m, H-3b), 2.20 (1H, brd, *J* = 12.0 Hz, H-5), 1.60 (1H, m, H-6a), 2.52 (1H, m, H-6b), 1.55 (1H, m, H-7a), 1.90 (1H, m, H-7b), 2.05 (1H, m, H-8), 1.83 (1H, m, H-9), 2.75 (1H, dd, J = 17.0, 7.0 Hz, H-11a), 2.30 (1H, dd, *J* = 17.0, 10.5 Hz, H-11b), 2.65 (1H, q, *J* = 7.2 Hz, H-14), 6.32 (1H, d, *J* = 1.8 Hz, H-15), 7.20 (1H, d, *J* = 1.8 Hz, H-16), 0.95 (3H, d, *J* = 7.2 Hz, H-17), 9.65 (1H, s, H-19), 10.02 (1H, s, H-20), 3.82 (3H, s, 18-OMe); ^{13}C NMR (100 MHz, CD_3OD) δ_C (ppm): 31.8 (C-1), 18.5 (C-2), 32.5 (C-3), 60.7 (C-4), 48.7 (C-5), 23.4 (C-6), 32.2 (C-7), 37.0 (C-8), 42.5 (C-9), 50.2 (C-10), 22.3 (C-11), 147.5 (C-12), 122.4 (C-13), 31.5 (C-14), 110.5 (C-15), 140.6 (C-16), 17.5 (C-17), 174.2 (C-18), 198.5 (C-19), 208.1 (C-20), 53.0 (OMe).

Cell lines and culture

HL-60 (human promyelocytic leukemia), HeLa (human cervical adenocarcinoma), MCF-7 (human breast adenocarcinoma), and LLC (lewis lung carcinoma) cells were obtained from the American Type Culture Collection (ATCC). The cells were maintained in RPMI or IMDM (GibcoBRL, Grand Island, NY) with 10% fetal bovine serum (FBS) supplemented with 2% penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Cytotoxic activity assay

The cancer cell lines were maintained in RPMI 1640 or IMDM that included L-glutamine (GIBCO) with 10% FBS (GIBCO) and 2% penicillin–streptomycin (GIBCO). Cells were cultured at 37 °C in a 5% CO_2 incubator. Cytotoxic activity was measured using a modified MTT assay (Lee et al., 2011a). Viable cells were seeded in the growth medium into 96-well microtiter plates (1×10^4 cells/well) and were incubated at 37 °C in a 5% CO_2 incubator. The test sample was dissolved in DMSO and was adjusted to final sample concentrations ranging from 1 to 50 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to <0.1%. After standing for 24 h, the test sample was added to each well. The same volume of medium with 0.1% DMSO was added to the control wells. After 48 h the test sample was added, MTT reagent was added to the each well (final concentration, 5 μg/mL). Four hours later, the plate was centrifuged for 5 min at 1500 rpm, the medium was removed, and the resulting formazan crystals were dissolved with DMSO. The optical density (OD) was measured at 570 nm using a Titertek microplate reader (Multiskan MCC/340, Flow, Middleboro, MA). The IC_{50} value was defined as the concentration of sample which reduced absorbance by 50% relative to the vehicle-treated control.

Caspase-3 activity

Caspase-3 enzyme activity was measured by proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AFC by counting on a fluorescence plate reader (Twinkle LB970 microplate fluorometer, Berthold Technologies, Bad Wildbad, Germany). HL-60 cells (1×10^5 cell/well) were treated with active compound at concentration ranging from 1 to 30 μM. After incubation for 24 h, cells were harvested and washed with cold PBS. The pellets were lyzed using 15 μL of lysis buffer [10 mM Tris-HCL (pH 8.0), 10 mM EDTA, 0.5% Triton X-100] at room temperature for 10 min, and then placed on ice; 100 μL of assay buffer [100 mM Hepes (pH 7.5), 10 mM dithiothreitol, 10% (w/v) sucrose, 0.1% (v/v) Chaps, 0.1% (v/v) BSA], and 10 μL of substrate solutin (200 μM substrate in assay buffer) were added. After incubation at 37 °C for 1 h, fluorescence was measured with an excitation at 370 nm and an emission at 505 nm (Lee et al., 2011a).

Western blotting analysis

HL-60 cells (5×10^5 cells/ml) were treated with tested compounds for 24 h at 37 °C. Cell lysates were prepared in 100 μL of lysis buffer (Sigma, St. Louis, MO) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). Insoluble material was removed by centrifugation at 14 000 rpm for 10 min. Then, the protein contents in the supernatant were measured using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). The protein extracts (50 μg/well) were separated by SDS-PAGE and then transferred onto PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBS-T) at 4 °C overnight and incubated with primary antibodies at room temperature for 1.5 h. The membranes were washed three times with TBS-T and blotted with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1.5 h, followed by washing three times in TBS-T. Immunoreactive proteins were visualized by an enhanced chemiluminescence procedure according to the protocol of the manufacturer (Santa Cruz Biotechnology, Santa Cruz, CA) and exposed to X-ray films. Protein contents were normalized by reprobing the same membrane with anti-β-actin antibody. For β-actin detection, previously used membranes were soaked in stripping buffer (Gene Bio-Application Ltd., Hanagid, Israel) at room temperature for 20 min.

Results and discussion

Five diterpenoids were isolated and identified as phanginin I (**1**), phaginin A (**2**), phanginin D (**3**), phanginin H (**4**), and phanginin J (**5**) (Figure 1) based on NMR spectroscopy and by comparing with published data (Yodsaoue et al., 2008; Zhang et al., 2012). Cancer cells were seeded in 96-well plates, then incubated for 4 h and were treated with the isolates (**1**–**5**) at various concentrations (0–50 μM). The inhibitory process was assessed by using an MTT assay (Lee et al., 2011a). As shown in Table 1, compounds **1**–**4** showed effective inhibition against HL-60 cells with the IC_{50} values of 16.4 ± 1.5, 19.2 ± 2.0, 11.7 ± 1.6, and 22.5 ± 5.1 μM, meanwhile compound **5** showed weak cytotoxic activity with an IC_{50} value of 46.9 ± 4.7 μM. Except for the weak inhibitory effect of compounds **4** and **5** (IC_{50} > 50 μM), the other compounds **1**–**3** exhibited cytotoxic activity against HeLa cells with IC_{50} values of 28.1 ± 3.6, 37.2 ± 3.4, and 22.7 ± 2.8 μM. Additionally, compound **4**

Figure 1. Chemical structure of diterpenoids **1–5** from seeds of Vietnamese *C. sappan.*

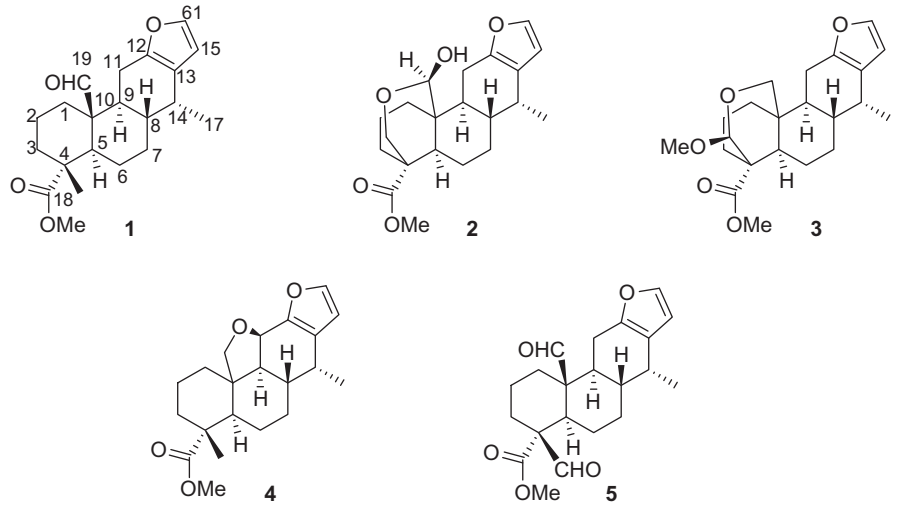

Table 1. Cytotoxic activity of compounds **1–5** from the seed of *C. sappan*.

	IC_{50} values (μM)[a]			
Fractions/compounds	HL-60	HeLa	MCF-7	LLC
CH_2Cl_2 fraction[b]	46.9 ± 10.5	117.5 ± 12.6	>200	>200
EtOAc fraction[b]	58.7 ± 7.1	68.4 ± 5.9	117.6 ± 5.6	>200
n-BuOH fraction[b]	>200	>200	>200	138.8 ± 8.6
1	16.4 ± 1.5	28.1 ± 3.6	>100	>100
2	19.2 ± 2.0	37.2 ± 3.4	>100	>100
3	11.7 ± 1.6	22.7 ± 2.8	>100	>100
4	22.5 ± 5.1	>50	>100	42.5 ± 5.1
5	46.9 ± 4.7	>50	>100	>100
Adriamycin[c]	2.8 ± 0.4	5.6 ± 0.5	2.2 ± 0.1	1.8 ± 0.3

[a]The inhibitory effects are represented as giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments (mean ± SD).
[b]In μg/mL
[c]Positive control.

showed weak inhibitory effect against LLC cancer cell line with an IC_{50} value of 42.5 ± 5.1 μM.

Caspase-3 is a cytosolic protein that normally exists as 32 kDa inactive precursor, and is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis. The activity of caspase-3 was measured by the proteolytic cleavage of Ac-Asp-Glu-Val-Asp-8-amino-4-trifluoromethylcoumarin in HL-60 cells for 12, 24, and 48 h (Av-DEVD-AFC) (Lee et al., 2011a) after treatment with compound **3** (1, 10, and 30 μM). As a result, the caspase-3 activity was increased by 1–5-folds in a dose- and time-dependent manner, as compared with the control (Figure 2).

The activity of caspase-3 was measured by the proteolytic cleavage of Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC), a fluorogenic substrate, after treatment with compound 3 (1–30 μM) in HL-60 cells for 24 h. Phanginin D (**3**) evidently induced proteolytic cleavage of procaspase-3 into its active form, and increased the caspase-3 activity from 10 to 30 μM. In the immunoblotting experiment using the antibody against PARP, the proteolytic cleavage of PARP by caspase-3 was used to assess the caspase-3 activation. PARP was found to be clearly cleaved from the intact form (116 kDa) into fragments (major fragment, 89 kDa) by treatment with compound 3 (30 μM) for 24 h (Figure 3).

The heartwood of *C. sappan* is a common remedy in traditional medicine and possesses diverse biological activities. Isolated compounds from the heartwood inhibited the growth of several cancer cell lines (Kim et al., 2005; Lee et al., 2011b; Liang et al., 2013). However, this is the first report on the cytotoxic activity of the constituents of Vietnamese *C. sappan* seeds in HL-60 and some other cell lines. Treatment with one of the active compounds, phanginin D (**3**), induced apoptosis thereby inhibiting the growth of HL-60 cancer cells. Activation of caspase-3 is required for several typical hallmarks of apoptosis and is indispensable for apoptotic chromatin condensation and DNA fragmentation in cell types examined, HL-60 (Figure 2). It remains unclear as to whether internucleosomal DNA fragmentation is a prerequisite for apoptosis (Schulze-Osthoff et al., 1994; Thornberry & Lazebnik, 1998), however, it is clear that caspase activation and subsequent PARP cleavage should be regarded as necessary for the consequent serial events of apoptosis (Oberhammer et al., 1993; Thornberry & Lazebnik, 1998). Our findings that the increment of caspase-3 activity and cleavage of procasepase-3 and PARP were induced by

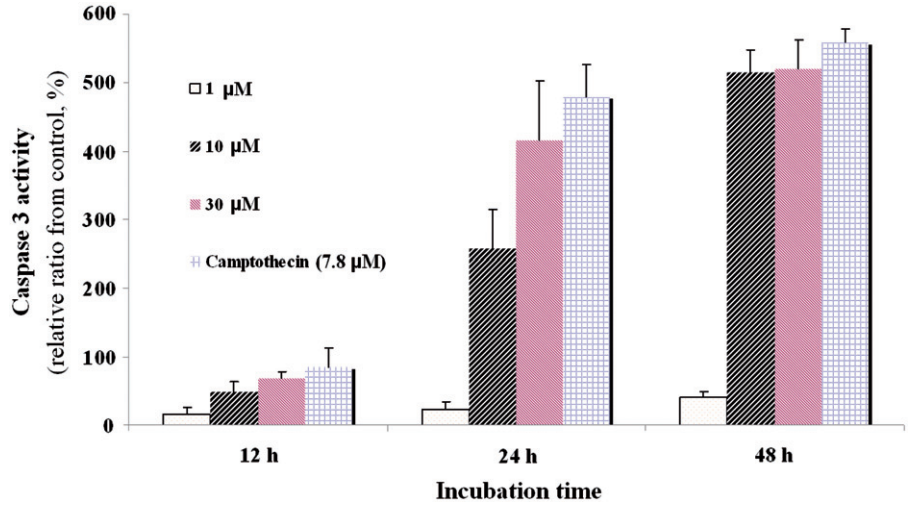

Figure 2. The increment of caspase-3 activity by compound **3** in HL-60 cells. After 12 h, 24 h, and 48 h incubation with the **3**-treated HL-60 cells, the cell lysates were incubated at 37 °C with caspase-3 substrate (Ac-DEVD-AFC) for 1 h. The fluorescence intensity of the cell lysates was measured to determine the caspase-3 activity. The blank group was used as 0.1% DMSO-treated cells; camptothecin (7.8 μM) was used as a positive control. Data are presented as the mean ± SD of results from three independent experiments.

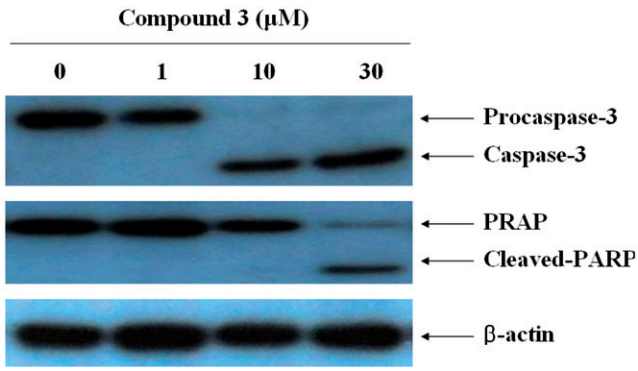

Figure 3. The induction of caspase-3 activation and PARP degradation by compound **3** in HL-60 cells. Western blot analysis of caspase-3 and PARP protein levels after exposure to compound **3**. HL-60 cells were treated with compound **3** at the indicated concentrations. Protein (50 μg) from each sample was resolved by SDS-PAGE (10% (w/v) polyacrylamide gel).

treatment with compound **3** imply that the death of the HL-60 cells may be induced principally by the apoptotic activity of this compound.

Conclusion

The present study demonstrates that active diterpenoid from *C. sappan* seeds induced HL-60 apoptosis via promoted caspase-3 pathway. This finding suggests that the natural deterpenoids from the seed of *C. sappan* are interesting potential candidates for the development on cytotoxic agents. Future studies focusing on cell signaling and biological significant of active compounds induced apoptosis are planned.

Declaration of interest

The authors have declared that there is no conflict of interest. This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under Grant number 106.05-2011.52.

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