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Isolation of the Cytotoxic Constituent Deoxypodophyllotoxin from the Leaves of *Juniperus chinensis*

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Deoxypodophyllotoxin was isolated as a cytotoxic constituent from ethanol extract of the leaves of *Juniperus chinensis* by assay-guided fractionation.

Key words : cytotoxic activity, HeLa cell, assay-guided fractionation, *Juniperus chinensis*

Introduction

The biodiversity of Malaysian plant resources offers about 15,000 species of higher plants. Most of them remain to be subjected to bioactivity-directed chemical studies though only one tenth

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phytochemical studies on the bioresources, the authors screened 24 samples of 13 plant species growing in Malaysia for cytotoxic activity against human cervical carcinoma cell line (HeLa) and found that the ethanol extract of leaves of *Juniperus chinensis* showed the strongest activity[1].

This paper describes the bioassay-guided isolation of a cytotoxic constituent of the plant and its identification with deoxypodophyllotoxin.

Materials and Methods

Plant material

Leaves with stems of *Juniperus chinensis* were collected from the ornamental growth in the Universiti Pertanian Malaysia (UPM) campus in January 1995. The plant taxonomic identification was done by Mr. Anthonysamy Sivarimuthu of the Department of Biology, UPM, and the botanical specimen was deposited in the department.

Spectral analyses

^{1}H- and ^{13}C-NMR spectra were taken by a Varian VXR-500 instrument and MS spectra were measured with a JEOL SX-102A mass spectrometer. IR and UV spectra were obtained by a Nicolet 710 FT-IR and a Shimadzu UV-3000

Cytotoxicity assay

Test Cells : HeLa cells (human cervical adenocarcinoma, RCB0007)[2] were obtained from RIKEN Cell Bank, Japan. The defrosted cells were transferred into a 15ml sterile centrifuge tube (Sumitomo Bakelite Co., Japan) containing 10ml of culture medium (CM). The cell suspension was centrifuged at 1500rpm for 5min. The supernatant was removed by aspiration and the remaining cell pellet was suspended in 1ml of CM. After washing twice with PBS (phosphate buffered saline), the cell suspension was transferred into a sterile 25cm² flask (Sumitomo Bakelite Co., Japan) containing 5ml of CM, and incubated at 37°C under 5% CO_2 atmosphere. The confluent

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cells were harvested.

Culture medium (CM) : 5.2 g of Roswell Park Memorial Institute Media (RPMI-1640, Gibco, USA)[3] dissolved in 450ml of sterilized water, 50ml of fetal calf serum (FCS) (Filtron, USA), 1ml of 5000μg/ml streptomycin, and 1ml of 5000IU/ml penicillin were mixed and sterilized by passing through a 0.22μm millipore membrane filter with a bottle filter system (Corning Coster Corp., USA).

Cytotoxicity test : An ethyl acetate solution of test material was diluted 50 times with ethanol and the ethanol solution was diluted twice with DMSO. The DMSO solution was diluted 10 times with RPMI-1640 medium to prepare a test solution of the highest concentration. One hundred μl each of the test solution of the highest concentration was added into one well on the first and second rows of a 96 well microplate (12×8 wells, Sumitomo Bakelite Co., Japan). The solution in the well of the second row was serially diluted two-fold with RPMI-1640 medium to prepare the test solution in the following wells. Each sample was prepared in duplicate. The plate was placed in a 5% CO_2 incubator at 37°C during preparation of the test cell suspension. The trypsinized confluent HeLa cells were suspended in 3ml of CM. The cell number in the suspension was adjusted to 1×10^6 cells/ml with CM by couting cells in a Neubauer's haemocytometer under a microscope. One hundred μl of the cell suspension was then added into each well of the plate already prepared with test solutions. The plate was placed in a 5% CO_2 incubator at 37°C. After incubation for 3 days (72 hours), the medium was blotted out with paper towels by shaking the plate gently. The cells were fixed with 100μl of 3.7(v/v) % formaldehyde in saline solution (formal saline) at room temperature for at least 20 minutes. After washing with tap water, the cells were stained with 1 to 2 drops of 20% crystal violet solution in methanol for 5 minutes. The minimum effective concentation (MEC) of the test material was determined by observing stained cells, and the 50% effective concentration (EC_{50}) was determined by counting the stained cells under a microscope.

Extraction of plant material

Two hundred g of leaves with stems were air dried overnight and macerated in 1 liter of 80% aqueous ethanol at room temperature for a week. After removing ethanol *in vacuo*, the remaining aqueous suspension was extracted succesively with petroleum ether (bp. 60–80°C), ethyl acetate, and butanol. A portion of each solvent extract was subjected to the cytotoxic assay.

Fractionation of the most active ethyl acetate soluble fraction

The ethyl acetate extract was developed with hexane-ethyl acetate (55 : 45) on Silica gel 60 PF_{254} (7747 ; E. Merck, Germany) packed in a quartz tubing. The silica gel column was divided into 5 portions, which were assayed for cytotoxic activity. The third fraction that was most active was subjected to column chromatography on Wakogel C-100 (Wako Pure Chemical Industries, Ltd., Japan) eluted with hexane-ethyl acetate (8 : 2) to give 7 fractions, the fifth of which was most active. The fraction was chromatographed on Wakogel C-300 (Wako Pure Chemical Industries, Ltd., Japan) packed in a stainless tubing eluted with hexane-ethyl acetate (75 : 25) at a flow rate of 1ml/min. The 6th of the 8 fractions was rechromatographed under the same conditions as above to give 7 fractions. The 4th fraction showed a major single spot, detected under UV_{254}, accompanied with a minor green spot visualized by a vanillin-sulfuric acid spray. These 2 spots were efficiently separated by passing the fraction through a SEP-PAK C_{18} cartridge (Waters Associates, USA) with 90% aqueous methanol to give a pure cytotoxic compound (**1**).

Results and Discussion

Isolation of a cytotoxic compound (**1**)

From 76.8 g of the residue of 80% ethanol extract of 200 g of leaves with stems of *Juniperus chinensis*, 13.6 g of ethyl acetate soluble fraction (MEC 0.03μg/ml) was obtained. Dry column chromatography of the ethyl acetate soluble fraction on silica gel PF_{254} (Merck) gave 1.6 g of the most active fraction (MEC 0.01μg/ml) among the 5 fractions. Column chromatography of this fraction on Wakogel C-100 eluted with hexane-ethyl acetate (8 : 2) gave 0.35 g of the most active fraction (MEC 0.01μg/ml), which was further chromatographed twice on Wakogel C-300 with hexane-ethyl acetate (75 : 25) at a flow rate of 1 ml/min to give 27mg of the active compound accompanied with green material. Removal of the green material by passing the mixture through a SEP-PAK C_{18} cartridge (Waters Associates, USA) resulted in the isolation of the active compound (**1**) (12mg, 0.0062% yield from the plant material).

Identification of the cytotoxic compound (**1**).

Compound **1** showed cytotoxic activity against HeLa cell with a MEC of 0.004μg/ml.

The molecular formula, $C_{22}H_{22}O_7$, was assigned to compound **1**, $[\alpha]_D^{25}$ −66.6° (*c* 0.6257, MeOH), based on the molecular ion peak at 398.1367 (Calcd. 398.1366) of EIHIMS. Compound **1** showed UV absorption bands (λ_{max}, MeOH) at 289 nm (ε 9.63×10³) and 293nm (ε 9.64×10³), and IR (KBr) absorption bands at 1778 (five membered lactone carbonyl), 1589, 1503, 1226, 1127, 998, and 943 cm⁻¹. Its EIMS spectrum displayed fragment peaks at *m/z* 398 (M^+, 100 %), 383 (5.7), 353 (4.4), 339 (4.6), 323 (3.1), 322 (2.1), 283 (5.1), 282 (3.7), 252 (2.4), 230 (7.8), 199 (5.8), 181 (20.7), 173 (17.2), and 168 (8.7%). Computer-search for this fragmentation pattern in the commercial library in NIST (National Institute of Science and Technology, USA) picked up that of deoxypodophyllotoxin, $C_{22}H_{22}O_7$.

All signals of ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) were assigned to every carbon and hydrogen of deoxypodophyllotoxin as shown in Table 1.

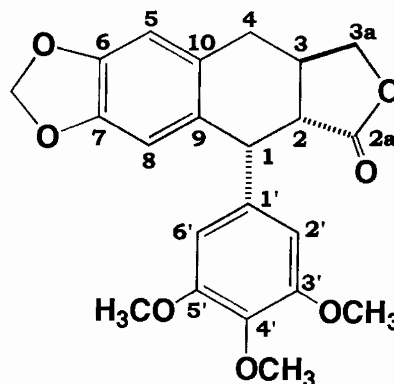

Fig. 1 Structure of deoxypodophyllotoxin.

Table 1 Assingnment of protom and carbon resonances of compound **1**

Hydrogen No.	δ_H	Carbon No.	δ_C	δ_C[4]
1-H	4.58	C-1	43.7	43.7
2-H	2.71	C-2	47.5	47.4
3-H	2.71	C 2a	174.9	174.6
3a-Ha	4.44	C-3	32.7	32.7
3a-Hb	3.92	C-3a	72.0	72.0
4-Ha	2.75	C-4	33.1	33.1
4-Hb	3.05			
5-H	6.65	C-5	108.5	108.1
		C-6	147.0	146.8
		C-7	146.7	146.5
8-H	6.50	C-8	110.4	110.3
		C-9	130.6	130.5
		C-10	128.2	128.1
		C-1'	136.3	136.0
2'-H	6.33	C-2'	108.2	108.1
		C-3'	152.5	152.3
		C-4'	136.9	136.9
		C-5'	152.5	152.3
6'-H	6.33	C-6'	108.2	108.1
$-OCH_2O-$	5.91, 5.93	$-OCH_2O-$	101.2	101.0
3'-OCH_3	3.73	3'-OMe	56.2	56.2
5'-OCH_3	3.73	5'-OMe	56.2	56.2
4'-OCH_3	3.79	4'-OMe	60.7	60.6

Compound **1** was, therefore, identified as deoxypodophyllotoxin. Deoxypodophyllotoxin is a well known cytotoxic compound which occurs widely in a variety of plant species, *e. g.*, *Anthriscus sylvestris*[5], *Austrocedrus chilensis*[6], *Biota orientalis*[7], *Brusera morelensis*[8], *B. permollis*[9], *Diphylleia cymosa*[10], *D. grayi*[10], *D. sinensis*[11], *Dysosma veitchii*[12], *Hernandia codigera*[13], *H. guianensis*[14], *H. ovigera*[15], *Hyptis tomentosa*[16], *Juniperus bermudiana*[17], *J. communis*[18], *J. phoenicea*[19], *J. sabina*[20], *J. thurifera*[21], *Kaempferia gelanga*[7], *Libocedrus pulmosa*[22], *Podophyllum emodi*[23], *P. hexandrum*[24], *P. peltatum*[25], *P. pleianthum*[26], *P. versipelle*[10], *Polygala macradenia*[27], *P. polygama*[28], *Thuja occidentalis*[29], and *Thujopsis dolabrata*[30].

To our knowledge, this is the first report of isolating the compound from *Juniperus chinensis*.

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細胞毒性化合物，Deoxypodophyllotoxin の *Juniperus chinensis* 茎葉からの単離と同定

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マレーシアなど熱帯地方において，庭木としてよく植栽されている *Juniperus chinensis* の茎葉のエタノール抽出物から HeLa 細胞に対する毒性を指標として，細胞毒性化合物 Deoxypodophyllotoxin を単離した．この化合物の HeLa 細胞に対する MEC は，0.004 μg/mℓであった．