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Bioassay-guided Isolation of Deoxypodophyllotoxin, the Cytotoxic Constituent of *Juniperus chinensis*

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Abstract – The ethanol extract from the leaves of *Juniperus chinensis* was found to be cytotoxic towards HeLa cells. Bioassay-guided fractionation of the EtOAc soluble faction directed by the microtitration cytotoxic assay revealed that the cytotoxic compound was deoxypodophyllotoxin. All the tumour cell lines tested (KU8112F-chronic mylogeneous leukemia, TK 10-renal carcinoma, UACC 62-melanoma and CEM-SS - T-lymphoblastic leukemia) were found to be susceptible to deoxypodophyllotoxin, however, the minimum effective concentration (MEC) required to reduce the cell population by 100 percent was different between cell lines.

Key words – *Juniperus chinensis*, Deoxypodophyllotoxin, Cytotoxicity

Introduction

Juniperus chinensis L. (Cupressaceae) is widely cultivated as an ornamental plant in South East Asia. The leaves have been reported to contain polyphenols similar to those found in the pine trees (Ibata *et al.*, 1984). Some of the flavonoids such as sciadopitysin, amentoflavone, sequoiaflavone, cupressuflavone, amentoflavone and hinokiflavone have been isolated from the leaves (Sheeja *et al.*, 1989). Three antifungal sesquiterpenes 8-acetoxyelemol, 8-hydroxyelemol, and hinokiic acid were isolated from the n-hexane fraction of methanol extract of the leaves of *Juniperus chinensis* var *pyramidalis* (Ohashi *et al.*, 1994). Studies on the chemical constituents of the acetone fraction of leaves extract has afford- ed umbelliferone, an unusual compound 2-3(3,4-methylenedioxyphenyl) propane-1,3-diol, thirteen lignans, sixteen diterpenes, a norditerpene and a secoditerpene (Fang *et al.*, 1992; 1993; Lee *et al.*, 1995). Seven novel abietanes and four known abietanes isolated from the ethyl acetate extract (Lee *et al.*, 1994).

We have reported that the antitumour-promoting and cytotoxic activities were present in the leave extract of *Juniperus chinensis* (Ali *et al.*, 1996). In this paper, we describe the bioassay-guided isolation, and characterization of the cytotoxic constituent that has never been reported in previous studies on the chemical constituents of *Juniperus chinensis*.

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Materials and methods

Plant material – The leaves of *Juniperus*

chinensis L. were collected within the Universiti Putra Malaysia (UPM) campus. Mr. Anthonysamy Sivarimuthu of the Department of Biology, UPM, identified the herbarium voucher specimen (MM-3) which was deposited at the Department of Biotechnology.

Extraction – The leaves were air-dried overnight and 200 g soaked in 1 liter of 80% aqueous ethanol at room temperature for a week. After the ethanolic solution was evaporated under reduced pressure, the remaining aqueous suspension was extracted successively with petroleum ether, ethyl acetate and butanol. Subsequently, each of the solvent extracts were assayed for cytotoxicity .

Cytotoxicity assay – The HeLa cell line (cervical adenocarcinoma) was obtained from RIKEN Cell Bank, Japan. Other cell lines, i.e. UACC-62 (melanoma), CEM-SS (T-cell lymphoblastic leukemia), KU812F (chronic myelogeneous leukemia) and TK-10 (renal carcinoma), were obtained from the National Cancer Institute, Maryland, USA. The cells were cultured and maintained in growth medium as described earlier (Ali *et al.*, 1996). Cytotoxicity was determined by performing the microtitration assay as described by Shier (1983). Briefly, each well was added with 100 µl of varying concentrations of the *Juniperus chinensis* extracts prepared from the stock solutions by serial dilution in RPMI-1640 medium. Subsequently, each well was filled with 100 µl of cell suspension in complete growth medium at $1\text{-}2\times10^4$ cells/ml except for CEM-SS which was added at 1×10^5 cells/ml. Controls containing only untreated cells were included for each sample. The assay for each concentration of extract was performed in triplicate and the culture plates were incubated at 37℃ with 5% (v/v) CO_2 for three to four days. Cytotoxicity was determined as the minimum effective concentration (MEC) of extract that killed all the cells in each well. Based on our previous report (Ali *et al.*, 1996), the HeLa cell line was chosen for the purpose of bioassay-guided isolation because of the strong cytotoxicity of the *Juniperus* extract towards this cell line.

Fractionation of the most active fraction (ethyl acetate soluble) – The ethyl acetate extract (13.60 g) was developed with hexane-ethyl acetate (55:45) on Silica gel 60 PF_{254} (7747; E. Merck, Germany) packed in a quartz column. The silica gel column was divided into 5 portions, which were assayed for cytotoxicity. The third fraction (1.6 g most active) was subjected to column chromatography on Wakogel C-100 (Wako Pure Chemical Industries, Ltd., Japan) and eluted with hexane-ethyl acetate (8:2) to give 7 fractions, with the fifth fraction showing the strongest activity. The fifth fraction (0.35 g) was chromatographed on Wakogel C-300 (Wako Pure Chemical Industries, Ltd., Japan) packed in a stainless steel column and eluted with hexane-ethyl acetate (75:25) at a flow rate of 1ml/min. The 6th (0.15 g) of the 8 fractions was rechromatographed under the same conditions as above to give 7 fractions. The 4th fraction (0.027 g) showed a major single spot, detected under UV254, accompanied with a minor green spot visualised by a vanillin-sulfuric acid spray. These 2 spots

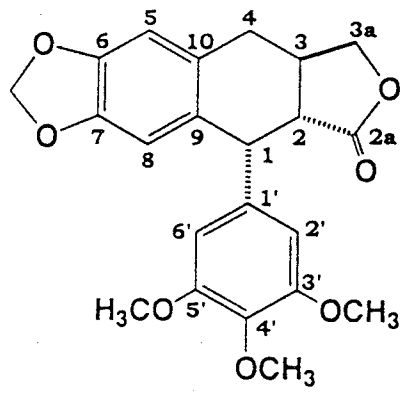

Fig. 1. Structure of deoxypodophyllotoxin.

were efficiently separated by passing the fraction through a SEP-PAK C18 cartridge (Waters Associates, USA) with 90% aqueous methanol to give a pure cytotoxic compound (12.4 mg).

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 using a Nicolet 710 FT-IR and a Shimadzu UV-3000 spectrometer, respectively. Specific rotation was measured by a Jasco DIP-360 polarimeter.

Results and Discussion

The molecular formula, $C_{22}H_{22}O_7$, was assigned to compound 1, $[\alpha]^{25}$-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 ($\in 9.63\times10^3$) and 293 nm ($\in 9.64\times10^3$), and IR (KBr) absorption bands at 1778 (five membered lactone carbonyl), 1589, 1503, 1226, 1127, 998, and 943 cm^{-1}. The EIMS spectrum of compound 1 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%). A computer-search for this fragmentation pattern in the commercial library in NIST (National Institute of Science and Technology, USA) matched this pattern that of deoxypodophyllotoxin, $C_{22}H_{22}O_7$ (Fig. 1).

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. Compound 1 was, therefore, identified as deoxypodophyllotoxin.

Deoxypodophyllotoxin was significantly cytotoxic towards all the cell lines tested (CEM-SS, 0.01 μg/ml; TK-10, 0.01 μg/ml; KU 812F, 0.04 μg/ml) particularly HeLa and UACC-62 (0.004 μg/ml). Deoxypodophyllotoxin is a well known cytotoxic compound which occurs widely in a variety of plant species particularly the *Podophyllum* (Liu *et al.*, 1979; Jackson and Dewick, 1981; Jackson and Dewick, 1985; Broomhead and Dewick, 1990; Kutney *et al.*, 1991) and *Juniperus* (Tammami *et al.*, 1977; Cairnes *et al.*, 1980; Markkanen *et al.*, 1981; San Feliciano *et al.*, 1989; San Feliciano *et al.*, 1990) species.

Table 1. Assignment of proton and carbon resonances of compound 1

Hydrogen No.	δ_H	Carbon No.	δ_C	δ_C*
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

*Fonseka *et al.* (1980)

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