생 약 학 회 지
Kor. J. Pharmacogn.
33(3) : 207 ~ 210 (2002)

Cytotoxic Compounds from *Croton cascarilloides*

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베트남산 *Croton cascarilloides*의 세포독성 물질

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Abstract – The methanol extract from the root of *Croton cascarilloides* Raeusch. was primarily evaluated for cytotoxic activity in the cultured human lung cancer cell line (A459) and showed cytotoxic potential with ED_{50} value of 5.98 μg/ml. Bioassay-guided fractionation of the root extract resulted in 3-acetyl aleuritolic acid, rubiadin-1-methyl ether, and julocrotine. The structures of the compounds were elucidated from the combination of spectroscopic data and references. In addition, the ^{13}C-NMR assignments of rubiadin-1-methyl ether were revised.

Key words – *Croton cascarilloides*, Euphorbiaceae, 3-*O*-Acetyl-D-friedoolean-14-en-28-oic-acid (3-acetyl aleuritolic acid), Rubiadin-1-methyl ether, Julocrotine, Cytotoxicity.

Under a collaboration program to discover new antitumor agents from Vietnamese medicinal plants, methanol extracts of 84 selected plants were primarily evaluated for cytotoxic activity against the cultures of human lung cancer cell line A549.[1)] Among the active extracts, the methanol extracts from the root and leaves of the plant *Croton cascarilloides* (Euphorbiaceae) showed significant cytotoxic activity against A549 cell line. The methanol extract was partitioned into chloroform and water. Chloroform fraction showed more potent cytotoxic activity than water one against A549 cells.[2)] *C. cascarilloides* is a popular medicine plant in Vietnam with the local name *Cu den la bac*. *C.* The root and stem of *cascarilloides* have been used as blood tonics, and in treatment of fever.[3)] To our knowledge, no report on biological and chemical study on this plant have been published so far. In this paper, we report on isolation of three compounds from the plant, their structural determination and cytotoxic activity.

MATERIALS AND METHODS

FAB mass spectra were recorded with a JMS-HX-110/110A tandem mass spectrometer (JEOL, Tokyo, Japan) using a JMS-DA 9000 data system. Melting points were measured on an electrothermal melting point apparatus and not corrected. Optical rotation was measured on a JASCO DIP-370 digital polarimeter. NMR experiments were carried out on Brucker 600 MHz spectrometer using TMS as reference.

Plant materials – The roots of *C. cascarilloides* were collected on February, 2001 at Mt. Tamdao in Vinh Phu, Vietnam. This plant was identified by Dr. Ngo Van Trai in National Institute of Materia Medica, Hanoi, where the voucher specimen of the plant was deposited.

Cytotoxicity assay – Cytotoxicity of the samples was measured using SRB assay.[1)] The ED_{50} value (a concentration that inhibits the cell growth by 50% relative to a vehicle-treated control) was calculated using regression analysis methods. The experiments were done in triplicate and expressed as average values.

Extraction and Isolation – The powdered root of *Croton cascarilloides* (750 g) was extracted with 95% methanol in water for 3 times (8 hrs each) and filtrated. The dark brown residue (36 g, F78) of the methanol extract was partitioned between $CHCl_3$ and H_2O to give a $CHCl_3$ layer (F78A) (16 g), a H_2O layer (F78B) and an insoluble interface (F78C) (9 g).

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The chloroform fraction (78A), which was the most cytotoxic (ED_{50} 2.55 μg/ml), was chromatographed over a silica gel column (5 × 40 cm) using a gradient of hexane and dichloromethane (80:20→0:100) and dichloromethane : methanol : water (70:10:0.5→70:30:10) to give 12 fractions. The eluents were detected by TLC (silica gel, 0.25 mm, Merck, Germany). The chromatogram was sprayed with 10% H_2SO_4, followed by heating. The seventh fraction (F78A7, 10 g, ED_{50} 0.5 μg/ml), which exhibited the cytotoxic activity, was then rechromatographed over a silica gel column (3 × 40 cm) with hexane/acetone/methanol to yield fifteen fractions. Fraction (78A7C, 1.1 g) and 78A7F were chromatographed over silica gel with hexane : acetone (5:1), and recrystallized successively from hexane : acetone (1:1) and aceton to give compound **1** as a white powder, mp. 293-294°C (50 mg), Rf: 0.71 (Hexane : EtOAc, 3:2), and compound **2** as a yellow needles (9.7 mg), mp. 263-264°C. Fraction 78A7L (2 g) was rechromatographed over a silica gel column (1.5 × 30 cm) with hexane : dichloromethane : methanol (6:3:1.2) to yield ten fractions. The solid from the fourth sub-fraction (78A7L4) was washed with ethyl acetate and purified further by semi-preparative reverse-phase HPLC (CBM-10A, Shimadzu, Kyoto, Japan). Julocrotine **3** (t_R : 10.73 min, 10 mg) was separated on a Water Spherisorb S5 ODS2 column (10 × 250 mm) with methanol : water (68:32) as a mobile phase (3 ml/min; UV detection at 254 nm).

3-acetyl aleuritolic acid (**1**) - 3-*O*-acetyl-D-friedoolean-14-en-28-oic-acid. M.w: 498.745 ($C_{32}H_{50}O_4$), $[\alpha]^{26.5}_D$=18.6 (*c*, 0.0215, $CHCl_3$), mp. 293–294°C. Positive FAB-MS (in $CHCl_3$ with NBA (Na), *m/z*: 543 $[M+2Na-H]^+$, 521 $[M+Na]^+$. ^1H-NMR (600 MHz, $CDCl_3$), δ : 1.03 (1H, H-1a), 1.61 (2H, H-1b, H-2 a), 1.66 (1H, H-2b), 4.47 (1H, *dd, J* = 5.2, 10.9 Hz, H-3), 0.88 (1H, H-5), 1.49 (1h, H-6a), 1.62 (1H, H-6b), 1.31 (1H, *d, J* = 12.96, H-7a), 1.97 (1H, *d, J* = 12.96, H7b), 1,42 (1H, H-9), 1.48 (1H, H-11a), 1.69 (1H, H-11b), 1.60 (1H, H-12a), 1.77 (1H, H-12b), 5.52 (1H, *dd, J* = 3.24, 7.8 Hz, H15), 1.92 (1H, *dd, J* = 3.3, 14.46 Hz, H-16a), 2.37 (1H, *dd, J* = 8.04, 14.24, H16b), 2.27 (1H, *dd, J* = 2.8, 14.34 Hz, H-18), 1.09 (1H, H-19a), 1.24 (1H, H-19b), 1.06 (1H, H-21a), 1.16 (1H, H-21b), 1.44 (1H, H22a), 1.69 (1H, H-22b), 0.85 (3H, H-23),0.88 (3H, H-24), 0,95 (6H, H-25, H-26), 0.92 (3H, H-27), 0.94 (3H, H-29), 0.91 (3H, H-30), 0.87 (1H, H-5), 2.04 (3H, $\underline{CH_3}$COO-).

^{13}C-NMR (150 MHz, $CDCl_3$), δ : 15.63 (C-25, CH_3), 16.58 (C-24, CH_3), 17.30 (C-11, CH_2), 18.73 (C-6, CH_2), 21.27 ($\underline{CH_3}$COO-, CH_3), 22.46 (C-27, CH_3), 23.45 (C-2, CH_2), 26.19 (C-26, CH_3), 27.94 (C-23, CH_3), 28.63 (C-30, CH_3), 29.30 (C-20, C), 30.68 (C-22, CH_2), 31.28 (C-16, CH_3), 31.83 (C-29, CH_2), 33.65 (C-21, CH_2), 33.31 (C-12, CH_2), 35.32 (C-19, CH_2), 37.29 (C-13, CH_2), 37.37 (C-1, C), 37.67 (C-4, C), 37.92 (C-10, C), 39.03 (C-8, C), 40.73 (C-7, CH_2), 41.36 (C-18, CH), 49.06 (C-9, CH), 51.51 (C-17, C), 55.58 (C-5, CH), 80.86 (C-3, CH), 116.86 (C-15, CH), 160.53 (C-14, C), 170.97 ($CH_3\underline{C}$OO-, C), 184.46 (C-28, C).

Rubiadin-1-methyl ether (**2**) – ^1H-NMR (600 MHz, DMSO-d_6) and ^{13}C-NMR (150 MHz, DMSO-d_6): as in Table 1, mp. 263-264°C. EI-MS (rel. int.), *m/z*: 268 $[M]^+$ (100%), 269 $[M+H]^+$ (16), 253 (43), 251 (20), 239 (24), 181 (11), 152 (12), 139, 76 (8).

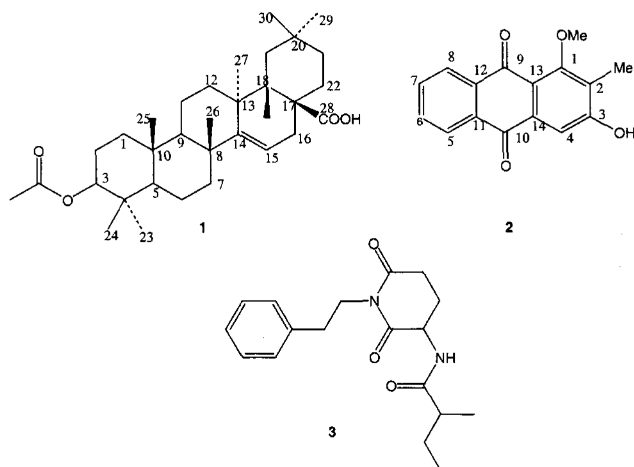
Julocrotine, 2-[*N*-(2-methylbutanoyl)]-N-phenylethyl-glutamide (**3**) – Positive FAB-MS (in $CHCl_3$): *m/z*: 317.0 $[M+H]^+$, with NBA (Na): 339.1 $[M+Na]^+$. ^1H-NMR ($CDCl_3$, 300 MHz) δ = 2.72 (2H, *m*, H-4), 2.51 (1H, *m*, H-5), 1.71 (1H, *m*, H-5), 4.49 (1H, *m*, H-6), 6.34 (1H, *d, J* = 5.04 Hz, H-7), 2.23 (1H, *dt, J* = 6.88, 1.26 Hz), 1.48 (1H, *m*, H-10), 1.71 (1H, *m*, H-10), 0.95 (3H, t, *J* = 7.41 Hz), 1.17 (1H, d, 6.88 Hz, H-12), 4.01 (2H, *m*, H-13), 2.82 (2H, *m*, H-14), 7.23 (2H, t, H10, H-20), 7.20 (2H, d, H17, H-19), 7.28 (1H, t, H18), 172.25 (C-1).

^{13}C-NMR ($CDCl_3$, 75 MHz) δ = 171.40 (C-3), 31.30 (C-4), 138.55 (C-5), 129.37 (C-6), 129.15 (C-7), 177.25 (C), 43.32 (C-9), 27.61 (C-10), 12.20 (C-11), 17.70 (C-12), 42.03 (C-13), 34.33 (C-14), 138.55 (C-15), 129.37 (C-16), 128.85 (C-17), 126.98 (C-18).

RESULTS AND DISSCUSSION

Compound **1** – The ^1H- and ^{13}C- NMR spectra of compound 1 showed a typical structural pattern of a triterpene with an acetyl group and a double bond. The ^{13}C- and DEPT spectra showed 3 of quarternary signals at δ 184.96, 170.97 and 160.53 ppm corresponding to the carboxylic carbon acid (C-28), the acetoxy carbon and the double bond carbon at C-14. The presence of one acetoxy group in compound 1 was revealed by a sharp 3H singlet at δ_H 2.04 and at δ_C 170.97 (*s*). Chemotaxonomic review of the Croton plants and comparison of the NMR data led to the confirmation that compound **1** was 3-acetyl aleuritolic acid, the same compound reported previously.[4)]

This compound is known to occur in many species of Euphorbiaceae family such as *Aleurites montana, Cnidoscolus vitifolius, Croton cajucara, C. urucurana, C. megalocarpus, Jatropha macrorhiza, Maprounea africana, Sapium sebiferum,* and *Phytolacca americana.* Compound **1** was showed



to have tumor-inhibitory properties toward the P-388 lymphocytic leukemia cells.[5] Recently, some similar triterpenoids have been shown to inhibit the catalytic activity of HIV-1 reverse transcriptase.[6]

Compound 2 – The spectral data (^{13}C-NMR) were nearly identical with those of rubiadin-1-methyl ether described in the literature,[7] except for the chemical shift value of the carbon atom at position 11 and 12 and the methyl group at position 2. Those discrepancies seemed to be resulted from a misinterpretation in the region of the spectra. Assignments of the ^{1}H- and ^{13}C-signals were achieved by using NMR measurements of HMBC for a long range $^{2}J_{H\text{-}C}$ and $^{3}J_{H\text{-}C}$ and HMQC for direct $^{1}J_{H\text{-}C}$ (Table 1). A correlation of the signal due to the H-5 (8.09 ppm) with C-7, C-12 and C-10 in the HMBC spectrum indicated the overlapped quarternery ^{13}C signal of the carbon 12 having the same chemical shift of δ 134.51 as those of C-7. The analysis of the NMR data confirmed that compound 2 was rubiadin-1-methyl ether, and its ^{13}C-NMR assignments were revised as described in Table 1 below.

The compound **2** has been isolated from Rubiaceae family (*Coprosma, Damnacanthus, Hymnodictyon, Morinda, Neonauclea, Pentas* and *Prismatomeris* genera), also from Gesneriaceae family (*Rhunchotechum*) and Menispermaceae family (*Tinomiscium*). This is the first report on the occurrence of the anthraquinone compound on Euphorbiaceae family as well as from the *Croton* genus. Compound **2** was previously reported to have high molluscicidal activity,[8] antiyeast and cytotoxic activity,[9] weak cardiotoxic activity[10] and antimalarial activity.[11]

Compound 3 – The compound **3** (Julocrotine) showed a FABMS spectral quasimolecular ion peak at *m/z* 317.0 [(M+H)]$^{+}$ in NBA and 339.1 [(M+Na)]$^{+}$ in NBA (Na) mode. The ^{1}H-NMR spectrum showed the presence of five protons at the aromatic field. These protons were determined to be due to the five protons of a substituted benzene ring. The aromatic signals were composed of one triplet signal at δ 7.23 for protons H-16 and H-20, one doublet signal at δ 7.20 for protons H-17 and H-19 and another triplet signal at δ 7.28 for protons H-18. The ^{13}C-NMR spectrum showed suitably the presence of a

Table I. NMR assignments of rubiadin-1-methyl ether (**2**)

Position	NMR assignments (ppm)		HMBC		Ref.[7]
	^{13}C	^{1}H	^{2}J	^{3}J	^{13}C
1	160.64	–	–		161.5
2	126.22	–	–		127.1
3	161.71	–	–		163.5
4	109.08	7.5 s	133.75, 161.71	117.91, 126.22, 182.64	110.2
5	126.06	8.09 d, 7.5 Hz		134.51, 182.64	126.8
6	133.33	7.82 t, 7.3 Hz		126.66	134.1
7	134.51	7.88 t, 7.3 Hz		134.51, 126.06	135.3
8	126.66	8.14 d, 7.5 Hz		133.33, 132.08, 180.21	127.5
9	180.21	–		–	180.8
10	182.64	–		–	183.6
11	132.08	–		–	135.5
12	134.51	–		–	132.9
13	117.91	–		–	117.8
14	133.75	–		–	134.6
1-OMe	60.61	3.8 s		160.64	61.4
2-Me	9.05	2.16 s	126.22	160.64, 161.71	9.9

Table II. Cytotoxic activity of the isolated compounds against B16 cells.

Compd.	ED_{50} (μg/ml)
1	>20
2	>20
3	10.84
Eptoposide	0.03

benzene ring by four signals at δ 138.55 (C-5), 129.37 (C-16 and C-20), 128.85 (C-17 and C-19) and 126.98 (C-18). Three nonhydrogenated peaks at δ 177.25, 172.25 and 171.40 were initially assigned to the amide-like carbons (CO-N). By analyzing of molecular weight and ^{1}H- and ^{13}C-NMR data, the presence of two nitrogen atoms were proposed. Literature search confirmed that compound **3** is identical with julocrotine, a glutarimide alkaloid,[12)] and has been isolated from *Croton membranaceus*,[13)] *C. humilis* [14)] species, and also from some other *Julocroton* species. This is the first time compound **3** was isolated from *C. cascarilloides* species.

CYTOTOXIC ACTIVITY

Cytotoxicity data of **1**, **2**, and **3** are summarized in Table 2. Compound **3** showed a moderate cytotoxic activity against B16 cell, a murine melanoma cells. Compound **2**, did not showed cytotoxic activity for the cells, even though that was reported to have cytotoxic activities against some other cells.[9)]

ACKNOWLEDGEMENT

One of us, N. M. Cuong is grateful to KOSEF (Korea Science and Engineering Foundation) for the Postdoctoral Fellowship for Foreign Researchers. We thank to Dr. Ngo Van Trai (National Institute of Materia Medica, Hanoi) for collecting and identifying plant sample.

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(Received April 20, 2002)

초 록 – 베트남산 *Croton cascarilloides* 뿌리의 메탄올 추출물을 인체 폐암세포인 A549에 대한 세포독성을 실시한 결과 유효함을 발견하였다(ED_{50}, 5.98 μg/ml). 세포독성을 측정하면서 유효들 물질을 분리하고 그들의 분광학적 데이터와 문헌치를 비교한 결과 이들은 각각 3-acetyl aleuritolic acid, rubiadin-1-methyl ether 및 julocrotine로 확인되었다.