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Cytotoxic and Apoptosis-Inducing Activities of Limonoids from the Seeds of *Azadirachta indica* (Neem)

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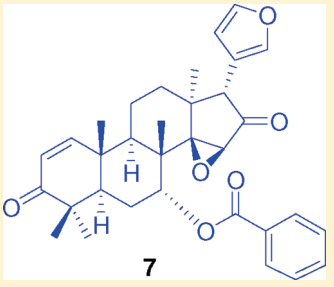
ABSTRACT: Thirty-five limonoids, including 15 of the azadiradione type (**1**−**15**), five of the gedunin type (**16**−**20**), four of the azadirachtin type (**21**−**24**), nine of the nimbin type (**25**−**33**), and two degraded limonoids (**34**, **35**), isolated from *Azadirachta indica* seed extracts, were evaluated for their cytotoxic activities against five human cancer cell lines. Seven compounds (**3**, **6**, **7**, **16**, **18**, **28**, and **29**) exhibited cytotoxic activity against one or more cell lines. Among these compounds, 7-deacetyl-7-benzoylepoxyazadiradione (**7**), 7-deacetyl-7-benzoylgeduin (**18**), and 28-deoxonimbolide (**28**) exhibited potent cytotoxic activity against HL60 leukemia cells with IC_{50} values in the range 2.7−3.1 μM. Compounds **7**, **18**, and **28** induced early apoptosis in HL60 cells, observed by flow cytometry. Western blot analysis showed that compounds **7**, **18**, and **28** activated caspases-3, -8, and -9 in HL60 cells. This suggested that compounds **7**, **18**, and **28** induced apoptotic cell death in HL60 cells via both the mitochondrial- and the death receptor-mediated pathways. Futhermore, compound **7** was shown to possess high selective cytotoxicity for leukemia cells since it exhibited only weak cytotoxicity against a normal lymphocyte cell line (RPMI 1788).



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The neem tree (*Azadirachta indica* A. Juss.; Meliaceae) is indigenous to India, but is widely cultivated throughout the tropics.[1] Since antiquity, various parts of the neem tree have been used for food, medicine, and insecticidal purposes, and many bioactive constituents including limonoids (tetranortriterpenoids) have been isolated and identified.[1−4] In the course of a search for naturally occurring bioactive compounds,[5,6] we have undertaken a detailed investigation of the limonoid constituents of the *n*-hexane[7] and defatted MeOH extracts[8] of *A. indica* seeds and have shown that some limonoids exhibit potent inhibitory effects against melanogenesis in B16 melanoma cells, against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, and against TPA-induced Epstein−Barr virus early antigen (EBV-EA) activation.[7,8] We have also reported that azadirachtin B (**21**) exerts a potent inhibitory effect on in vivo two-stage mouse skin carcinogenesis.[8] In a continuing study on the bioactivity of the limonoid constituents of *A. indica* seeds, the cytotoxicity of these compounds has been evaluated. We report the cytotoxic activities of 35 limonoids from *A. indica* seeds against five human cancer cell lines, namely, HL60 (leukemia), A549 (lung), AZ521 (stomach), SK-BR-3 (breast), and CRL1579 (melanoma). In addition, the induction of apoptosis in HL60 by 7-deacetyl-7-benzoylepoxyazadiradione (**7**), 7-deacetyl-7-benzoylgedunin (**18**), and 28-deoxonimbolide (**28**) and the mechanisms of the apoptotic cell death were investigated.

Thirty-five limonoids of the azadiradione (**1**−**15**), gedunin (**16**−**20**), azadirachtin (**21**−**24**), and nimbin (**25**−**33**) types and two degraded limonoids (**34**, **35**) were isolated from *n*-hexane[7] and defatted MeOH extracts[8] of *A. indica* seeds. The cytotoxic activities of compounds **1**−**35** and two reference anticancer drugs, cisplatin and 5-fluorouracil, were evaluated against five human cancer cell lines by means of a thiazoyl blue tetrazolium bromide (MTT) assay, and the results are summarized in Table 1. Seven compounds, **3**, **6**, **7**, **16**, **18**, **28**, and **29**, exhibited IC_{50} values of 1.7−9.9 μM against one or more cancer cell lines. Thus, compound **3** was active against HL60 and CRL1579 cells, **7** against HL60 and AZ521 cells, **16** against HL60 and SK-BR-3 cells, **28** against HL60, A549, AZ521, and SK-BR-3 cells, and **6**, **18**, and **29** against HL60 cells. In particular, the cytotoxic activities of compound **3** against HL60 and CRL1579 cells, **7** against HL60 and AZ521 cells, **16** against HL60 and SK-BR-3 cells, **18** against HL60 cells, and **28** against HL60, A549, AZ521, and SK-BR-3 cells were observed to be superior to those of the reference compounds, cisplatin and/or 5-fluorouracil, tested in the same assay.

On the basis of the results in Table 1, the following conclusions can be drawn about the structure−activity relationship of the compounds evaluated: (i) Azadiradione-type, gedunin-type, and nimbin-type limonoids possess in general potent or moderate cytotoxic activity. (ii) Azadirachtin-type and degraded limonoids possess relatively weak cytotoxic activity. (iii) Azadiradione-type and gedunin-type limonoids with an acetoxy group at the C-7α position are more active than those with a hydroxy group, as has been observed for **1** vs **2**, and **16** vs **17**. Furthermore, those compounds with a benzoyloxy group at the C-7α position were far more active than those with an acetoxy group, as observed for **3** vs **1**, **7** vs **6**, and **18** vs **16**. (iv) Gedunin-type limonoids with a 7α-acetoxy or a 7α-benzoyloxy group were more active than the

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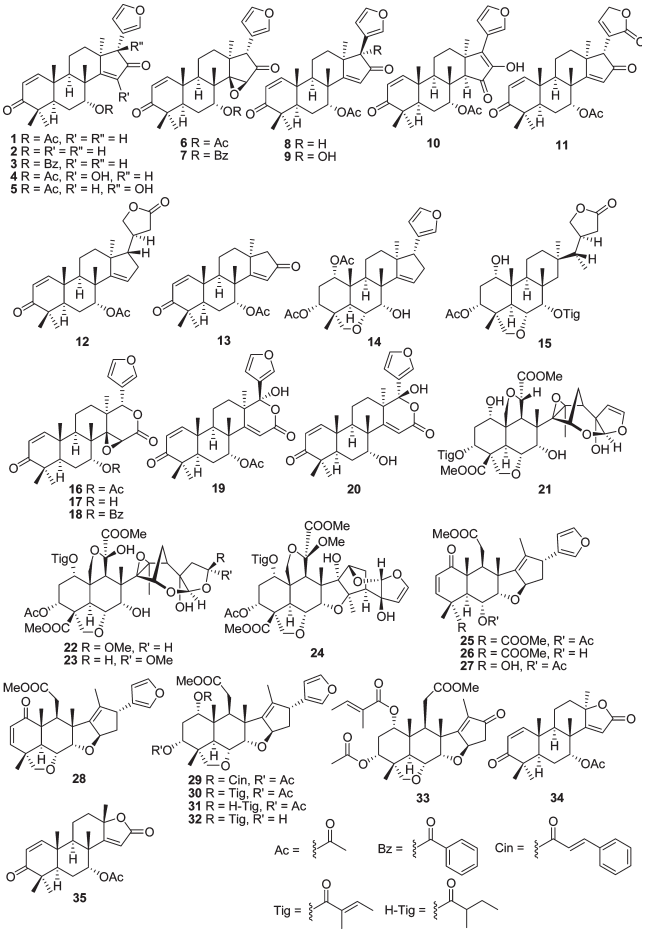

corresponding azadiradione-type limonoids against HL60 cells (**6** vs **1** and **18** vs **3**). (v) Defurylation at C-17 in azadiradione-type limonoids exerted almost no influence on the activity against HL60 cells (**1** vs **13**). (vi) Substitution of a conjugated cyclopentenone-type D-ring of **13** by an α,β-unsaturated γ-lactone-type D-ring (**34** and **35**) reduced the activity against HL60 cells. (vii) Hydroxylation at C-17(β) (**5**) or inversion of the stereochemistry at C-17 (**8**) of **1** exerted almost no influence on the activity against HL60 cells. However, inversion of the stereochemistry at C-17 along with the hydroxylation at C-17(α) (**9**) of **1** reduced the activity. Hydroxylation at C-15 (**4**) of **1** also reduced the activity against HL60 cells. (viii) A nimbin-type limnoid with a hydroxy group at the C-6α position is more active than that with an acetoxy group (**26** vs **25**). (ix) Substitution of a cinnamoyl group at C-1 by a dihydrotigloyl or a tigloyl group for nimbin-type limonoids reduced the activity against HL60 cells (**29** vs **31** vs **30**).

Compounds **7**, **18**, and **28** exhibited potent cytotoxic activities against HL60 cells (IC_{50} 3.1, 2.9, and 2.7 μM, respectively), and these were evaluated for their apoptosis-inducing activity using HL60 cells. HL60 cells were incubated with these test compounds for 8 and 24 h, and then the cells were analyzed by means of flow cytometry with annexin V−propidium iodide (PI) double staining. Exposure of the membrane phospholipid phosphatidylserine to the external cellular environment is one of the earliest markers of apoptotic cell death.[9] Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for phosphatidylserine expressed on the cell surface. Propidium iodide (PI) does not enter whole cells with intact membranes and was used to differentiate between early apoptotic (annexin V positive, PI negative), late apoptotic (annexin V, PI double positive), or necrotic (annexin V negative, PI positive) cell death. The ratio of early apoptotic cells (lower right) was increased after treatment with **7** in HL60 cells for 8 h (38.7% vs 11.3% of negative control) and 24 h (51.8% vs 6.3% of negative control), and that of late apoptotic cells (upper right) was increased after 24 h (15.5% vs 0.6% of negative control) (Figure 1A). In the case of compound **18**, the ratio of early apoptotic cells was increased after treatment for 24 h (41.4% vs 6.3% of negative control), and that of late apoptotic cells (upper right) was increased after 24 h (45.7% vs 0.6% of negative control) (Figure 1B). After treatment with compound **28**, the ratio of early apoptotic cells was increased after 8 h (10.9% vs 6.1% of negative control) and 24 h (18.7% vs 3.5% of negative control), and that of late apoptotic cells (upper right) was increased after 24 h (28.1% vs 2.9% of negative control) (Figure 1C). These results revealed that most of the cytotoxic activities of compounds **7**, **18**, and **28** against HL60 cells are due to inducing apoptotic cell death.

Caspases are known to mediate the apoptotic pathway.[10,11] Caspases-8 and -9, which are initiator caspases, appear to be the apical caspases activated in death receptor- and mitochondrial stress-induced apoptotic cell death, respectively. Initiator caspases are responsible for either directly or indirectly activating various effector caspases, including caspases-3, -6, and -7, which contain short prodomains. Effector caspases cleave a number of structural and regulatory proteins and are directly responsible for many of the apoptotic features, which are nuclear and cytoplasmic condensation, DNA fragmentation, cell membrane composition, and others.[12] In order to clarify the mechanisms by which compounds **7**, **18**, and **28** induce apoptotic cell death, activation of caspases-3, -8, and -9 was evaluated by Western blot analysis. The levels of procaspases-8, -9, and -3 diminished, and then the levels of cleaved caspases-8, -9, and -3 were increased after treatment with compounds **7**, **18**, and **28** (Figure 2). These results suggest that compounds **7**, **18**, and **28** induced apoptotic cell death via both the mitochondrial- and the death receptor-mediated pathways.

Compounds **7**, **18**, and **28** and cisplatin were then tested for their cytotoxicity against a normal lymphocyte cell line, RPMI 1788, and their selectivity index (SI) value,[13] which was determined by dividing the IC_{50} value for the normal cell line (RPMI 1788) by the IC_{50} value for the cancer cell line (HL60). As shown in Table 2, compounds **7**, **18**, and **28** exhibited SI values of 5.19, 0.69, and 1.00, respectively, greater than that of cisplatin (SI 0.45). In particular, compound **7** exhibited the highest selective cytotoxicity for leukemia cells since it exhibited only weak cytotoxicity for the RPMI 1788 cell lines.

From the results of the cytotoxicity evaluation of the limonoids from *A. indica* seed extracts, it appears that some of these may be valuable anticancer lead compounds. Furthermore, compound **7**, which induced apoptotic cell death in leukemia and displayed high selective toxicity against leukemia, may be especially promising in this regard. Although compounds **6**,[14,15] **16**,[16−18] and **28**[19,20] have been reported previously to show potent or moderate cytotoxicity against several cancer cell lines, the mechanisms of cell death were not explored.

EXPERIMENTAL SECTION

General Experimental Procedures. Nine limonoids, **2**, **14**, **20**, and **28**−**33**, were isolated from the *n*-hexane extract of *A. indica* seeds,[7] with another 29 limonoids, **1**, **3**−**13**, **15**−**19**, **21**−**27**, **34**, and **35**, isolated

Table 1. Cytotoxic Activities on Five Human Cancer Cell Lines of Limonoids from Neem (*Azadiracta indica* A. Juss.) Seed Extracts[a]

		IC_{50} (μM)[b,c]				
compound		HL60 (leukemia)	A549 (lung)	AZ521 (stomach)	SK-BR-3 (breast)	CRL1579 (melanoma)
azadiradione type						
1	azadiradione	14.7	>20	>20	15.9	>20
3	7-benzoylnimbocinol	5.3	>20	>20	>20	4.0
5	17-hydroxyazadiradione	12.4	>20	>20	>20	>20
6	epoxyazadiradione	9.3	>20	>20	12.3	>20
7	7-deacetyl-7-benzoylepoxyazadiradione	3.1	>20	8.4	>20	>20
8	17-epiazadiradione	12.6	>20	>20	>20	>20
9	17-epi-17-hydroxyazadiradione	>20	>20	>20	>20	19.1
11	azadiradionolide	>20	>20	>20	11.5	>20
13	desfuranoazadiradione	19.8	>20	>20	>20	>20
14	1,3-diacetylvilasinin	15.3	>20	14.8	>20	>20
gedunin type						
16	gedunin	5.9	>20	16.9	8.3	>20
17	7-deacetylgedunin	>20	>20	16.9	12.0	>20
18	7-deacetyl-7-benzoylgedunin	2.9	>20	>20	>20	>20
nimbin type						
25	nimbin		>20	>20	>20	15.9
26	6-deacetylnimbin	11.6	>20	>20	>20	>20
28	28-deoxonimbolide	2.7	9.3	2.4	1.7	14.2
29	ohchinin acetate	9.9	>20	>20	>20	>20
31	2′,3′-dihydrosalannin	17.1	>20	>20	>20	>20
reference compounds						
	cisplatin	4.2	>20	9.5	18.8	>20
	5-fluorouracil	9.1	>20	>20	>20	>20

[a] Cells were treated with compounds (1×10^{-4} to 1×10^{-6} M) for 48 h, and cell viability was analyzed by the MTT assay. [b] The IC_{50} value is the concentration of compound required to inhibit the growth of the cells by 50%. This was obtained based on triplicate assay results. [c] Compounds **2** (nimbocinol), **4** (15-hydroxyazadiradione), **10** (7-acetyl-16,17-dehydro-16-hydroxyneotrichilenone), **12** (20,21,22,23-tetrahydro-23-oxoazadirone), **15** (3-acetyl-7-tigloylvalasinin lactone), **19** (nimolicinol), **20** (7-deacetyl-17-epinimolicinol), **21** (azadirachtin B), **22** (vepaol), **23** (23-epivepaol), **24** (3-acetyl-11-methoxy-1-tigloylazadirachtinin), **27** (6-acetylnimbandiol), **30** (salannin), **32** (3-deacetylsalannin), **33** (17-defurano-17-oxosalannin), **34** (α-nimolactone), and **35** (β-nimolactone) exhibited IC_{50} values of >20 μM in all cell lines used.

Table 2. Cytotoxicities of Compounds 7, 18, and 28 and Cisplatin against Leukemia (HL60) and Normal Lymphocyte (RPMI 1788) Cell Lines[a]

		IC_{50} (μM)[b]		
compound		HL60 (leukemia)	RPMI1788 (normal lymphocyte)	SI[c]
7	7-deacetyl-7-benoylepoxyazadiradione	3.1	16.1	5.19
18	7-deacetyl-7-benzoylgedunin	2.9	2.0	0.69
28	28-deoxonimbolide	2.7	2.7	1.00
	cisplatin[d]	4.2	1.9	0.45

[a] Cells were treated with compounds (1×10^{-4} to 1×10^{-6} M) for 48 h, and cell viability was analyzed by the MTT assay. [b] IC_{50} based on triplicate assay results. [c] SI refers to the selectivity index, which was obtained by dividing the IC_{50} value for the normal cells by the IC_{50} value for the cancer cells. [d] Reference compound.

from the defatted MeOH extract of *A. indica* seeds.[8] Chemicals and reagents were purchased as follows: fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI) 1640 medium, antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin), and nonessential amino acid (NEAA) from Invitrogen Co. (Auckland, New Zealand), Dulbecco's modified Eagle's medium (D-MEM), Eagle's minimum essential medium (MEM), and MTT from Sigma-Aldrich Japan Co. (Tokyo, Japan), and rh Annexin V/FITC kit (Bender MedSystems) from Cosmo Bio Co. Ltd. (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

Cell Cultures. Cell lines HL60 (leukemia), A549 (lung), AZ521 (stomach), SK-BR-3 (breast), and CRL1579 (melanoma) were obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan). A normal cell line, RPMI 1788 (lymphocytic), was obtained from Health Science Research Resources Bank (Osaka, Japan). Three cell lines, HL60, SK-BR-3, and CRL1579, were grown in RPMI 1640 medium, while A549

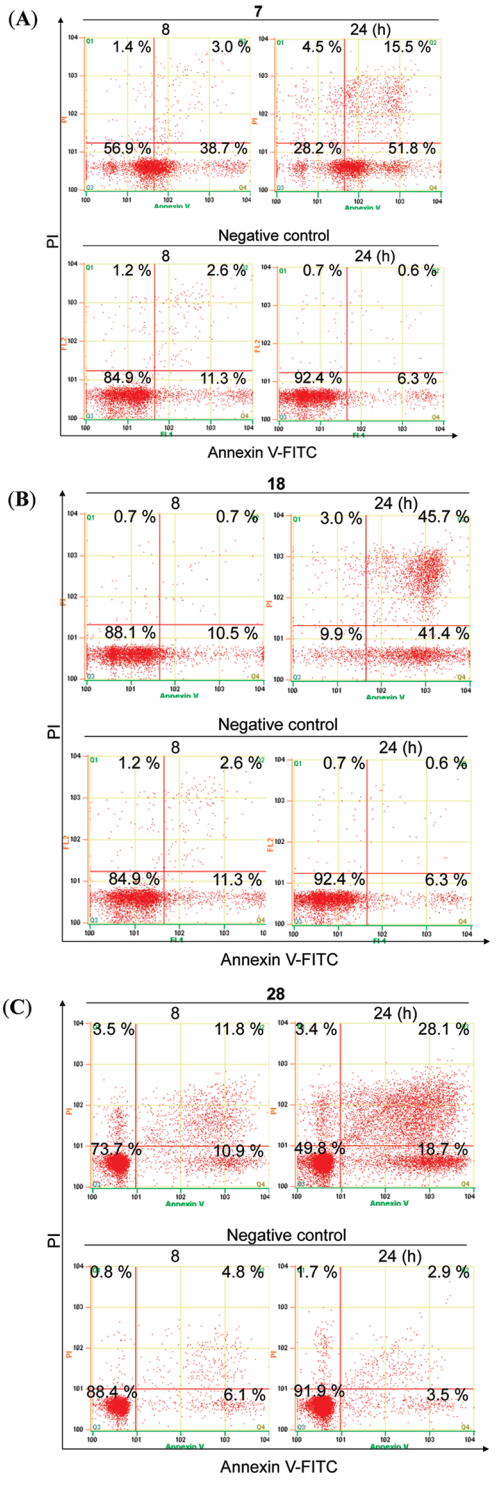

Figure 1. Compounds **7**, **18**, and **28** induced apoptosis against HL60 cells. (A) HL60 cells were cultured with compound **7** (30 μM) for 8 and 24 h. (B) HL60 cells were cultured with compound **18** (30 μM) for 8 and 24 h. (C) HL60 cells were cultured with compound **28** (30 μM) for 8 and 24 h. Each value is the mean of three experiments.

and AZ521 cell lines were grown in D-MEM and in 90% D-MEM + 10% MEM + 0.1 mM NEAA, respectively. The medium was supplemented with 10% FBS and antibiotics. Cells were incubated at 37 °C in a 5% CO_2 humidified incubator.

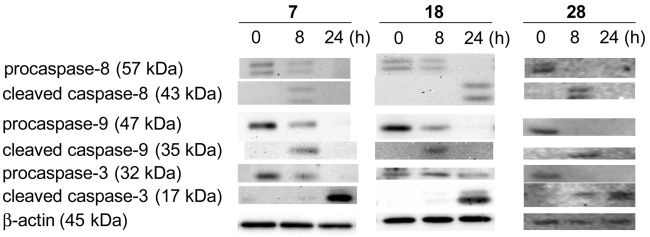

Figure 2. Western blot analysis of HL60 cells treated with 30 μM each of compounds **7**, **18**, and **28**. The results are from one representative experiment among three runs, which showed similar patterns to one another.

Cytotoxicity Assays. Cytotoxicity assays were performed according to methodology previously reported.[21,22] Briefly, the cell lines cited above [each 3×10^3 cells/well] were treated with test compounds for 48 h, and then MTT solution was added to each well. After incubation for 3 h, the blue formazan generated was solubilized with 0.04 M HCl in 2-propanol. The absorbances at 570 nm (top) and 630 nm (bottom) were measured with a microplate reader (Tecan Japan Co., Ltd., Kawasaki, Japan).

Annexin V−Propidium Iodide (PI) Double Staining. Annexin V−PI double staining was performed according to the method previously reported.[21] Apoptosis was detected using an rh annexin V/FITC kit. HL60 (1×10^5 cells) were exposed to each test compound (final concentration: 30 μM). To prepare the cell sample for flow cytometry, cells were washed with annexin-binding buffer and stained with annexin V−fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 10 min. Cell samples were analyzed using a flow cytometer (Cell Lab Quanta SC; Beckman Coulter K.K., Tokyo, Japan) with the FL1 and FL2 ranges for annexin V−FITC and −PI, respectively.

Western Blotting. Western blot analysis was performed according to the method previously reported.[21,22] Briefly, HL60 (4×10^5 cells) were exposed to test compound (final concentration: 30 μM) for 8 and 24 h. Cells were collected and lysed. Lysates of total protein were separated by 15% sodium dodecyl sulfate−polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with anticaspase-3 (Sigma-Aldrich Japan Co., Tokyo, Japan) and anticaspase-8, anticleaved caspase-3, anticaspase-9, and anti-β-actin (Cell Signaling Technology, Beverly, MA) primary antibodies at 4 °C overnight. The blots were then detected with enhanced chemiluminescence plus Western blotting detection system (GE Healthcare, Chalfont St. Giles, U.K.) .

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