The Melanogenesis-Inhibitory, Anti-Inflammatory, and Chemopreventive Effects of Limonoids in *n*-Hexane Extract of *Azadirachta indica* A. Juss. (Neem) Seeds

Toshihiro Akihisa^{1*}, Akitomo Takahashi¹, Takashi Kikuchi¹, Mio Takagi¹, Kensuke Watanabe¹, Makoto Fukatsu¹, Yukiko Fujita², Norihiro Banno², Harukuni Tokuda³ and Ken Yasukawa⁴

¹ College of Science and Technology, Nihon University (1-8-14 Kanda Surugadai, Chiyoda-ku, Tokyo 101-8308, JAPAN)

² Ichimaru Pharcos Company Ltd. (318-1 Asagi, Motosu-shi, Gifu 501-0475, JAPAN)

³ Department of Complementary and Alternative Medicine, R&D, Graduate School of Medical Science, Kanazawa University (13-1 Takara-machi, Kanazawa 920-8640, JAPAN)

⁴ School of Pharmacy, Nihon University (7-7-1 Narashinodai, Funabashi-shi, Chiba 274-8555, JAPAN)

Abstract: Seventeen limonoids (tetranortriterpenoids 1-17) were isolated from the *n*-hexane extract of *Azadirachta indica* (neem) seeds. The previously unidentified compound 16 was established by spectroscopy to be 17-defurano-17-oxosalannin. The effects of six compounds, 6 and 11-15, on melanogenesis in B16 melanoma cells was evaluated; 2 compounds, salannin (13) and 3-deacetylsalannin (15), exhibited marked inhibitory effects (70-74% reduction of melanin content at 25 µg/mL) with only minor cytotoxicity (79-85% of cell viability). Eleven compounds, 2, 3, 5, 6, and 9-15, were evaluated for inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (1.7 nmol/ear) in mice; all exhibited marked anti-inflammatory activity (ID₅₀ values 0.22-0.57 µmol/ear). In addition, compounds 6 and 11-16 exerted moderate inhibition (IC₅₀ values of 410-471 mol ratio/32 pmol TPA) of TPA-induced Epstein-Barr virus early antigen (EBV-EA) activation in Raji cells. The triacylglycerol fraction of the *n*-hexane extract contained oleic acid (50.2%) as the most predominant fatty acid constituent.

Key words: *Azadirachta indica*, neem seeds, limonoids, antimelanogenesis activity, anti-inflammatory activity, chemopreventive effect

1 INTRODUCTION

The neem tree (*Azadirachta indica* A. Juss.; Meliaceae) is indigenous to India, but is widely cultivated throughout the tropics¹⁾. Since antiquity, various parts of the neem tree have been used for food, medicine, and as insecticides, and many bioactive constituents including limonoids (tetranortriterpenoids) have been isolated and identified¹⁻⁴⁾. In the course of our search for naturally occurring bioactive compounds⁵⁾, we have undertaken a detailed investigation of the limonoid constituents of MeOH extracts of defatted *A. indica* seeds and have shown that some limonoids exhibited potent inhibitory effects on melanogenesis in B16 melanoma cells, on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, and on TPA-induced Epstein-Barr virus early antigen (EBV-EA) activation⁶⁾. We

have also reported that azadirachtin B exerts a potent inhibitory effect on *in vivo* two-stage mouse-skin carcinogenesis⁶⁾. In continuing our study of the constituents of *A. indica* seeds, we have investigated *n*-hexane extracts and isolated a new (16) and 16 known (1-15, 17) limonoids. We now report on their characterization and an evaluation of their inhibitory effects on melanogenesis in B16 melanoma cells, TPA-induced inflammation in mice, and TPA-induced EBV-EA activation.

2 EXPERIMENTAL PROCEDURES

2.1 General experimental procedures

Optical rotation was measured in EtOH on a JASCO

*Correspondence to: Toshihiro Akihisa, College of Science and Technology, Nihon University, 1-8-14 Kanda Surugadai, Chiyoda-ku, Tokyo 101-8308, JAPAN

E-mail: akihisa@chem.cst.nihon-u.ac.jp

Accepted August 3, 2010 (received for review July 29, 2010)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online

<http://www.jstage.jst.go.jp/browse/jos/>

P-1020 Digital Polarimeter. UV spectra, using a JASCO V-630Bio spectrophotometer, and IR spectra, using a JASCO FTIR-300E spectrometer, were recorded in EtOH and KBr disks, respectively. NMR spectra were recorded with a JEOL ECX-400 (^1H , 400 MHz; ^{13}C , 100 MHz) spectrometer in CDCl_3 with tetramethylsilane as an internal standard. ESIMS and high-resolution (HR)-ESIMS were recorded with the positive-ion mode on an Agilent 1100 LC/MSD TOF (time-of-flight) system. Silica gel (Silica gel 60, 220-400 mesh, Merck & Co. Inc., Darmstadt, Germany) and C_{18} silica (ODS; Chromatorex-ODS, 100-200 mesh; Fuji Silysia Chemical, Ltd., Aichi) were used for open column chromatography. Reversed-phase preparative HPLC was carried out on ODS columns (25 cm $\times$ 10 mm i.d.) with a mobile phase flow rate of 3.0 mL/min on a Capcell Pak AQ column (Shiseido Co., Ltd., Tokyo) with $\text{MeCN}/\text{H}_2\text{O}/\text{AcOH}$ at a ratio of 50:50:0.1 (HPLC system I) or 55:45:0.5 (HPLC system II), or on a TSK-A column (Toso Co., Ltd., Tokyo) with $\text{MeCN}/\text{H}_2\text{O}/\text{AcOH}$ (60:40:0.1) (HPLC system III) as the eluting solvent.

2.2 Materials and chemicals

The source of the *Azadirachta indica* A. Juss. (Meliaceae) seeds was described previously⁶⁾. Ten limonoids, azadiradione (**1**), 17-epiazadiradione (**2**), 17-hydroxyazadiradione (**3**), 17-epi-17-hydroxyazadiradione (**4**), azadiradionolide (**5**), desfuranoazadiradione (**7**), nimbin (**8**), 6-deacetylnimbin (**9**), 6-acetylnimbandiol (**10**), and nimolcinol (**17**), were used as reference compounds⁶⁾. Chemicals and reagents were obtained from the following sources: Fetal bovine serum (FBS) from Trace Scientific Ltd. (Melbourne, Australia), Eagle's minimal essential medium (MEM) from Gibco (Grand Island, NY, U.S.A.), arbutin (4-hydroxyphenyl β -D-glucopyranoside) from Tokyo Chemical Industry Co., Ltd. (Tokyo), TPA from ChemSyn Laboratories (Lenexa, KS, U.S.A.), thiazolyl blue tetrazolium bromide (MTT), indomethacin, and (all-*trans*)-retinoic acid from Sigma-Aldrich Japan Co. (Tokyo), and the EBV cell culture reagents and *n*-butanoic acid from Nacalai Tesque, Inc. (Kyoto). All other chemicals and reagents were of analytical grade.

2.3 Extraction and isolation

The seeds (2.05 kg) of *A. indica* were pulverized and extracted with *n*-hexane (2 L) under reflux (3 h) 3 times to yield an *n*-hexane extract (280 g)⁶⁾. A portion (176 g) of the *n*-hexane extract was subjected to column chromatography (CC) on a silica gel (1.76 kg), which gave a less-polar oily-substance (144 g; triacylglycerol) from the eluates of *n*-hexane and *n*-hexane-ethyl acetate (EtOAc) (8:2), and a more-polar semi-solid substance (27 g) from the eluates of EtOAc. The latter fraction was purified by the same chromatography system to yield a solid substance (18.4 g) which, upon further CC on a silica gel (500 g) with solvents

of increasing polarity [*n*-hexane-EtOAc (8:2 $\rightarrow$ 0:10)], yielded 9 fractions, A-I, listed in increasing order of polarity. Fraction D (3560 mg) was subjected to CC on a silica gel (150 g) to yield 5 fractions, D1-D5. Fraction D1 (2183 mg) was purified by CC on an ODS (100 g) [eluent: acetonitrile (MeCN)- H_2O (4:6 $\rightarrow$ 10:0)] to yield purified fraction D1 (525 mg). Preparative HPLC (system I) of fraction D1 yielded compounds **1** (16.3 mg), **2** (20.7 mg), **3** (257.8 mg), and **4** (14.7 mg). Fraction D2 (745 mg) was, after purification (382 mg) by CC on an ODS (31 g), subjected to preparative HPLC (system I) which yielded compounds **8** (5.0 mg), **9** (45.3 mg), and **17** (33.8 mg). Preparative HPLC (system I) of fraction D4 (137 mg) yielded compound **5** (8.4 mg). Fraction E (1174 mg) was passed through silica gel to yield five fractions, E1-E5. Preparative HPLC (system III) of fractions E1 (277 mg) and E2 (557 mg) yielded compounds **13** (76.6 mg) and **14** (30.1 mg), respectively. Fraction F (1945 mg) was passed through CC on a silica gel followed by on an ODS to obtain a purified fraction (624 mg) which, upon preparative HPLC (system II), yielded compounds **6** (183.0 mg), **7** (2.0 mg), **11** (15.6 mg), **12** (2.3 mg), and **13** (124.5 mg). Furthermore, fractions G (774 mg) and H (619 mg), upon purification by CC on a silica gel followed by ODS, yielded purified fractions G (88 mg) and H (92 mg). Preparative HPLC of the purified fractions G (system III) and H (system I) yielded compounds **15** (9.5 mg) and **16** (5.3 mg), respectively.

2.4 Identification and characterization

Ten compounds, **1-5**, **7-10**, and **17**, were identified by ^1H NMR and MS comparisons with reference compounds. Identification of the following six compounds was performed by ^1H NMR and MS comparison with compounds described in the literature: 1,3-diacetylvilasinin (**6**)⁷⁾, 28-deoxonimbolide (**11**)⁸⁾, ohchinin acetate (**12**)⁹⁾, salannin (**13**)¹⁰⁾, 2',3'-dihydrosalannin (**14**)¹¹⁾, and 3-deacetylsalannin (**15**)¹²⁾ (Fig. 1).

Compound **16** (17-defurano-17-oxosalannin) was a new compound, and its characterization was performed by spectroscopic methods. The ^{13}C , ^1H , and HMBC NMR spectroscopic data for compound **16** are shown in Table 1. Compound **16** was an amorphous solid; $[\alpha]_D^{22} + 24.7$ (c 0.35, MeOH); UV (EtOH) λ_{max} (log ϵ) 223 (3.64) nm; IR (KBr) ν_{max} 1736 (ester C=O), 1714 (conjugated cyclopentenone) cm^{-1} ; HR-ESIMS m/z 545.2678 (calculated for $\text{C}_{37}\text{H}_{37}\text{O}_4[\text{M} + \text{H}]^+$, 545.2691).

Fatty acid composition of the triacylglycerol fraction (less-polar oily-substance) of the *n*-hexane extract of *A. indica* seeds was determined as the methyl ester derivative as described previously¹³⁾.

2.5 Cell culture

B16 murine melanoma cells purchased from Riken Cell Bank (Tsukuba, Japan) were cultured in MEM supplement-

Fig. 1 Structures of compounds 1-17.

ed with 5% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified CO₂ controlled (5%) incubator.

2.6 Determination of cell proliferation

Cell proliferation was assessed using the MTT colorimetric assay. The protocol for this *in vitro* assay has been described⁽⁶⁾.

2.7 Assay of melanin content

Melanogenesis-inhibitory activity was assessed in B16 melanoma cells. The protocol for this *in vitro* assay has been described⁽⁶⁾.

2.8 Assay of TPA-induced inflammation ear edema in mice

For the protocol for this *in vivo* assay, refer to previous articles^{14, 15)}.

2.9 *In vitro* EBV-EA activation experiment

For the protocol for this *in vitro* assay, refer to previous

articles^{14, 15)}.

3 RESULTS AND DISCUSSION

3.1 Limonoid constituents

Seventeen limonoids, including 7 azadirones (1-7), 9 nimbins (8-16), and 1 gedunin (17), were isolated from the *n*-hexane extract of *Azadirachta indica* seeds (Fig. 1). Among these, 7 compounds, 6 and 11-16, were newly isolated in this study from the *n*-hexane extract whereas 10 other compounds, 1-5, 7-10, and 17, have also been isolated from the MeOH extract of defatted *A. indica* seeds⁽⁶⁾. As is expected from the non-polar nature of the *n*-hexane extract, most of the compounds isolated in this study were less-polar compounds that did not possess a polar hydroxyl group. Among the 17 compounds, compound 16 was previously unidentified, and its structure is described here.

The molecular formula of compound 16 was determined to be C₃₀H₄₀O₉ on the basis of its HR-ESIMS ([M + H]⁺, *m/z* 545.2678). The UV spectrum showed an absorption maxi-

mum at 223 nm, consistent with an α , β -unsaturated ketone system¹⁶⁾ while the IR spectrum indicated the presence of ester carbonyl (1736 cm^{-1}) and conjugated cyclopentenone (1714 cm^{-1}) groups^{6,17)}. The ^{13}C and ^1H NMR spectra (Table 1) indicated the presence of 8 methyls [three tertiary methyls (δ_{H} 1.03, 1.24, and 1.40), 3 vinyl methyls (δ_{H} 1.77, 1.82, 1.90), 1 acetate methyl (δ_{H} 2.00), and 1 oxymethyl (δ_{H} 3.36)], 4 methylenes including 1 oxymethylene (δ_{C} 77.8), 7 sp^3 methines of which 5 were oxymethines (δ_{H} 4.05, 4.14, 4.94, 5.01, 5.09), 1 sp^2 methine (δ_{H} 6.97), 7 sp^2 quaternary carbons, and 3 sp^3 quaternary carbons (δ_{C} 40.8, 42.7, 50.1). The presence of an oxymethyl (δ_{H} 3.36) and a vinyl methyl (δ_{H} 1.77) group, and a characteristic C-28-methylene group (δ_{H} 3.64 and 3.72, each d and $J = 7.4$ Hz), forming a C-6 ether linkage as in salannin (13), indicated 16 to be a salannin-type ring C-*seco*-limonoid¹⁰⁾. Moreover, the ^1H NMR data of 16 were almost superimposable with those of 13¹⁰⁾ except for the absence of the ^1H signals due to a furan-ring attached at C-17. Compound 16 exhibited, however, a ^{13}C signal (δ_{C} 206.0) due to a keto group located most probably at C-17 in ring-D. In the HMBC spectrum, diagnostic cross-correlations were observed between δ_{H} 1.77 (H-18) and δ_{C} 206.0 and between δ_{H} 2.87 (H-16 β) and δ_{C} 206.0, which confirmed the presence of a keto group at C-17 (Table 1). The above evidence, coupled with the analysis of ^1H - ^1H COSY, HMQC, and HMBC (Table 1) spectra, indicated the structure of 16 as methyl

Fig. 2 Major NOE correlations ($\leftrightarrow$) for compound 16¹⁸⁾

3 α -acetoxy-7 α , 15 β -epoxy-6 α , 4 α -epoxymethano-4,8-dimethyl-1 α -tigloyloxy-18,20,21,22,23,24-hexanor-11,12-*seco*-5 α -chol-13-en-17-one-11-carboxylate (17-defurano-17-oxosalannin). The relative configuration, deduced from NOESY (Fig. 2) and molecular modeling¹⁸⁾, fully supported the proposed structure. This seems to be the first report on the isolation of a limonoid with a degraded nimbine-type structure (without a furan-ring at C-17) from a plant.

3.2 Limonoid Bioactivity

Six compounds, 6 and 11-15, were evaluated for melanogenesis inhibition. Addition of each of these compounds (25 $\mu\text{g/mL}$) to B16 melanoma cell cultures inhibited mela-

Table 1 ^{13}C , ^1H , and HMBC NMR spectroscopic data (δ values; CDCl_3) for 17-defurano-17-oxosalannin (16)^{a)}

C no.	δ_{C}	δ_{H}	HMBC (H to C)	C no.	δ_{C}	δ_{H}	HMBC (H to C)
1	70.6 d	4.94 (t, 2.8)	3, 1'	16	45.9 t	2.47 (α , dd, 3.2, 16.9)	15
2	28.1 t	2.16 (α , dt, 2.5, 16.5)	10			2.87 (β , dd, 6.4, 16.9)	13, 15, 17
		2.27 (β , dt, 3.2, 16.5)	10	17	206.0 s		
3	71.2 d	5.01 (t, 2.8)	1, <u>OCOMe</u>	18	8.2 q	1.77 (d, 1.9)	13, 14, 17
4	42.7 s			19	15.3 q	1.03 (s)	1, 5, 9, 10
5	40.2 d	2.81 (d, 12.4)	4, 6, 7, 10, 19, 29	28	77.8 t	3.72 (α , br d, 7.4)	4, 29
6	72.3 d	4.05 (dd, 3.3, 12.4)	5, 7			3.64 (β , d, 7.4)	4, 5, 6, 29
7	84.7 d	4.14 (d, 3.3)	6, 7, 9	29	19.5 q	1.24 (s)	3, 4, 5, 28
8	50.1 s			30	16.3 q	1.40 (s)	7, 8, 9, 14
9	40.4 d	2.85 (dd, 3.2, 10.1)	12, 14	1'	166.5 s		
10	40.8 s			2'	128.8 s		
11	30.4 t	2.28 (dd, 3.2, 15.6)	8, 9, 10, 12	3'	137.5 d	6.97 (dq, 1.3, 7.3)	1', 4', 5'
		2.40 (dd, 10.1, 15.1)	8, 9, 10, 12	4'	14.4 q	1.82 (dd, 0.9, 7.3)	2', 3'
12	172.3 s			5'	11.9 q	1.90 (t, 1.2)	1', 2', 3'
13	134.0 s			<u>OCOMe</u>	170.4 s		
14	176.6 s			<u>OCOMe</u>	21.0 q	2.00 (s)	<u>OCOMe</u>
15	78.7 d	5.09 (sept-like, 2.2)	14	<u>OMe</u>	51.5 q	3.36 (s)	12

a) J values (Hz) determined are shown in parentheses.

Table 2 Melanogenesis inhibitory activities and cytotoxicities of compounds isolated from the *n*-hexane extract of *Azadirachta indica* seeds in B16 mouse melanoma cells

Melanogenesis inhibitory activity and cytotoxicity			
		Mean $\pm$ S.E. (%) ^{a)}	
	Compound	Melanin content ^{b)}	Cell viability ^{b)}
control	(100% DMSO)	100.0 $\pm$ 2.5	100.0 $\pm$ 0.7
6	1,3-Diacetyltilasinin	11.9 $\pm$ 0.2	31.8 $\pm$ 0.7
11	28-Deoxonimbolide	7.4 $\pm$ 0.1	1.9 $\pm$ 0.1
12	Ohchinin acetate	17.1 $\pm$ 0.1	62.5 $\pm$ 1.1
13	Salannin	30.4 $\pm$ 2.6	79.1 $\pm$ 1.7
14	2',3'-Dihydrosalannin	10.3 $\pm$ 0.5	30.6 $\pm$ 0.6
15	3-Deacetylsalannin	25.8 $\pm$ 0.8	85.3 $\pm$ 1.5
	Arbutin ^{c)}	77.4 $\pm$ 2.9	102.0 $\pm$ 1.5

a) Sample concentration determined was 25 μ g/mL. Melanin contents (%) and cell viability (%) were determined based on the absorbances at 420 and 570-620 nm, respectively, by comparison with those for DMSO (100%). The absorbances at 420 and 570-620 nm of DMSO were 0.523

b) Values of fourfold experiments. Concentration of DMSO in the sample solution was 5 μ L/mL.

c) Reference compound.

nogenesis, reducing the melanin content to 7.4-30.4% (Table 2). These compound exerted greater inhibitory effects than a reference arbutin (melanin content 77.4%) which has been recognized as a useful depigmentation compound for skin whitening in the cosmetic industry¹⁹⁾. Although the inhibitory effect of 4 compounds, **6**, **11**, **12**, and **14**, is likely due to their cytotoxicity (1.9-62.5% cell viability at 25 μ g/mL), 2 nimbin-type limonoids, **13** and **15**, showed remarkable inhibition of melanogenesis (melanin content 30.4 and 25.8% at 25 μ g/mL, respectively) with only minor cytotoxicity (cell viability 79.1 and 85.3%, respectively). The melanogenesis-inhibitory effects of 10 other compounds, **1-5**, **7-10**, and **17**, have previously been evaluated, and potent inhibition of melanogenesis with no or almost no cytotoxicity has been observed for 1 azadirone-type (**7**), 2 nimbin-type (**8**, **9**), and 1 gedunin-type (**17**) limonoid⁶⁾. Of the nimbin-type limonoids evaluated, those possessing a methyl carboxylate group at C-4 with a 2-en-1-one structure (**8**, **9**)⁶⁾ or an *O*-tiguloyl group at C-1 with a 6 α , 4 α -epoxymethano structure (**13**, **15**) exerted a potent melanogenesis inhibitory effect with quite low cytotoxicity.

Inhibition of TPA-induced inflammation in mice by eleven compounds, **2**, **3**, **5**, **6**, and **9-15**, was evaluated. The results are shown in Table 3 together with that of a commercially available anti-inflammatory drug, indomethacin. All of the compounds tested exerted potent inhibitory effects with ID₅₀ (50% inhibitory dose) values of 0.22-0.75 μ mol/ear, and were more potently inhibitive than indomethacin

(ID₅₀ 0.91 μ mol/ear). Four other compounds, **1**, **4**, **8**, and **17**, have previously been evaluated for their inhibitory effects against TPA-induced inflammation, and have been shown to possess potent inhibitory activity with ID₅₀ 0.20-0.94 μ mol/ear⁶⁾.

The inhibitory effects on TPA-induced EBV-EA were examined as a preliminary evaluation of the potential anti-tumor-promoting activity²⁰⁾ of 7 compounds, **6**, and **11-16**. The results are shown in Table 3, together with comparable data for retinoic acid, which has been shown to have chemopreventive activity in a number of tumors²¹⁾. All compounds tested maintained a high degree of viability (60%) in Raji cells even at 32 nmol (mol ratio of compound to TPA = 1000:1), indicating their low cytotoxicity at this high concentration. Each tested compound exhibited an inhibitory effect, with an IC₅₀ value (concentration of 50% inhibition with respect to positive control) of 370-471 mol ratio/32 pmol TPA. As such, these compounds were more potent than or almost equipotent to the reference retinoic acid (482 mol ratio/32 pmol TPA). Ten other compounds, **1-5**, **7-10**, and **17**, have also been reported to possess inhibitory activity against TPA-induced EBV-EA activation induced (IC₅₀ value 359-499 mol ratio/32 pmol TPA)⁶⁾. Since the inhibitory effects against EBV-EA activation have been demonstrated to closely parallel those against tumor promotion *in vivo*²⁰⁾, those compounds highly inhibitory against EBV-EA activation could be valuable anti-tumor promoters.

Table 3 Inhibitory effects of compounds from the *n*-hexane extract of *Azadirachta indica* seeds on TPA-induced inflammation in mice and on the induction of Epstein-Barr virus early antigen

Compound		Inhibition of inflammation		Percentage of EBV-EA induction ^{c)}				IC ₅₀ ^{d)}
		ID ₅₀ ^{a)}	95%CI ^{b)}	Concentration (mol ratio/TPA)				mol ratio/32 pmol TPA
				1000	500	100	10	
2	17-Epiazadiradione	0.56	0.50-0.62					
3	17-Hydroxyazadiradione	0.47	0.41-0.54					
5	Azadiradionolide	0.29	0.19-0.44					
6	1,3-Diacetylvilasinin	0.25	0.22-0.29	11.8 (60)	49.8	76.2	100	471
9	6-Deacetylnimbin	0.75	0.67-0.83					
10	6-Acetylnimbandiol	0.42	0.24-0.51					
11	28-Deoxonimbolide	0.36	0.25-0.52	10.5 (60)	49.2	76.6	100	469
12	Ohchinin acetate	0.23	0.19-0.29	5.9 (60)	47.8	73.6	100	452
13	Salannin	0.22	0.18-0.26	0.0 (60)	42.3	70.9	92.3	370
14	2',3'-Dihydrosalannin	0.40	0.36-0.45	0.0 (60)	43.9	71.6	93.8	410
15	3-Deacetylsalannin	0.57	0.40-0.81	0.0 (60)	45.2	72.5	96.7	420
16	17-Defurano-17-oxosalannin			0.0 (60)	45.7	73.5	95.2	418
	Indomethacin ^{e)}	0.91	0.76-1.09					
	Retinoic acid ^{e)}			15.3 (60)	49.3	76.3	100	482

a) 50% inhibitory dose.

b) 95% confidence intervals.

d) IC₅₀ represents the molar ratio to TPA that inhibits 50% of positive control (100%) activated with 32 pmol TPA.

c) Values represent percentages relative to the positive control value. TPA (32 pmol, 20 ng) = 100%. Values in parentheses are viability percentages of Raji cells.

e) Reference compound.

3.3 Fatty acid composition

The fatty acid composition of the triacylglycerol fraction of the *n*-hexane extract of *A. indica* seeds was determined as: myristic[14:0; mean $\pm$ standard deviation ($n=3$); $0.7 \pm 0.54\%$], palmitic (16:0; $17.6 \pm 0.21\%$), palmitoleic (16:1, $n-7$; $0.1 \pm 0.08\%$), stearic (18:0; $18.6 \pm 0.37\%$), oleic (18:1, $n-9$; $50.2 \pm 1.37\%$), *cis*-vaccenic (18:1, *cis*-11; $0.4 \pm 0.55\%$), elaidic (18:1, *trans*-9; $0.5 \pm 0.12\%$), linolelaidic (18:2, *trans*-9, *trans*-12; $0.2 \pm 0.07\%$), linoleic (18:2, *n*-6; $9.4 \pm 0.12\%$), α -linolenic (18:3, *n*-3; $0.2 \pm 0.05\%$), arachidic (20:0; $1.6 \pm 0.05\%$), behenic (22:0; $0.3 \pm 0.01\%$), erucic (22:1, *n*-9; $0.2 \pm 0.14\%$), and lignoceric (24:0; $0.3 \pm 0.01\%$) acids. This fatty acid profile is almost consistent with that reported in the literature²²⁾, but our *A. indica* seeds contained more oleic acid and less linoleic acid than those reported previously (oleic acid 40.1%; linoleic acid 21.3%)²²⁾.

From the results of our present and recent studies⁶⁾, it appears that salannin(**13**) and some other limonoid constituents of the *n*-hexane extract and the defatted MeOH extract⁶⁾ of *A. indica* seeds could be valuable as skin whitening agents, anti-inflammatory agents, and chemopreventive agents in chemical carcinogenesis. Although the anti-

inflammatory effect of sodium nimbinat against carrageenan-induced edema and HCHO-induced arthritis in rats has previously been reported^{4, 23, 24)}, our findings on the anti-inflammatory effects of various neem limonoids against TPA-induced inflammation in mice merit study in a clinical setting. In addition, the results of this and our recent studies⁶⁾ will be of value for increasing utilization of neem seed oil in the cosmetic and pharmaceutical fields.

ACKNOWLEDGEMENT

This work was supported, in part, by a Grant-in-Aid from the Japan Society for the Promotion of Science (No. 20590015), 2008-2010.

References and Notes

- 1) van der Nat, J. M.; van der Sluis, W. G.; de Silva, K. T.; Labadie, R. P. Ethnopharmacognostical survey of *Azadirachta indica* A. Juss (Meliaceae). *J. Ethnophar-*

- macol.* **35**, 1-24 (1991).
- 2) Biswas, K.; Chattopadhyay, I.; Banerjee, R. K.; Bandyopadhyay, U. Biological activities and medicinal properties of neem (*Azadirachta indica*). *Curr. Sci.* **82**, 1336-1345 (2002).
- 3) Akhila, A.; Rani, K. Chemistry of the neem tree (*Azadirachta indica* A. Juss.). *Fortschr. Chem. Org. Naturst.* **78**, 47-149 (1999).
- 4) Brahmachari, G. Neem-An omnipotent plant: A retrospection. *ChemBioChem* **5**, 408-421 (2004).
- 5) Akihisa, T.; Yasukawa, K. Anti-inflammatory and antiallergic properties of triterpenoids from plants. in *Bio-materials from Aquatic and Terrestrial Organisms*; (Fingerman, M.; Nagabhushanam R. ed.) Science Publ. Enfield. pp. 63-114 (2006).
- 6) Akihisa, T.; Noto, T.; Takahashi, A.; Fujita, Y.; Banno, N.; Tokuda, H.; Koike, K.; Suzuki, T.; Yasukawa, K.; Kimura, Y. Melanogenesis inhibitory, anti-inflammatory, and chemopreventive effects of limonoids from the seeds of *Azadirachta indica* A. Juss. (neem). *J. Oleo Sci.* **58**, 581-594 (2009).
- 7) Connolly, J. D.; Labbé, C.; Rycroft, D. S.; Taylor, D. A. H. Tetranortriterpenoids and related compounds. Part 22. New apotirucallol derivatives and tetranortriterpenoids from the wood and seeds of *Chisocheton paniculatus* (Meliaceae). *J. Chem. Soc., Perkin Trans. I* 2959-2964 (1979).
- 8) Kigodi, P. G. K.; Blaskó, G.; Thebtaranonth, Y.; Pezzuto, J. M.; Cordell, G. A. Spectroscopic and biological investigation of nimbolide and 28-deoxonimbolide from *Azadirachta indica*. *J. Nat. Prod.* **52**, 1246-1251 (1989).
- 9) Ochi, M.; Kotsuki, H.; Kataoka, T.; Tada, T.; Tokoroyama, T. Limonoids from *Melia azedarach* Linn. var. *Japonica* Makino. III. The structures of ohchinal and ohchinin acetate. *Chem. Lett.* 331-334 (1978).
- 10) Nakatani, M.; Zhou, J.-B.; Nakayama, N.; Okamura, H.; Iwagawa, T. Nimbolidins C-E, limonoid antifeedants from *Melia toosendan*. *Phytochem.* **41**, 739-743 (1996).
- 11) Yamasaki, R. B.; Klocke, J. A. Structure-bioactivity relationship of salannin as an antifeedant against Colorado potato beetle (*Leptinotarsa decemlineata*). *J. Agric. Food Chem.* **37**, 1118-1124 (1989).
- 12) Johnson, S.; Morgan, E. D. Comparison of chromatographic systems for triterpenoids from neem (*Azadirachta indica*) seeds. *J. Chromatogr. A* **761**, 53-63 (1997).
- 13) Akihisa, T.; Kojima, N.; Katoh, N.; Ichimura, Y.; Suzuki, H.; Fukatsu, M.; Maranz, S.; Masters, E. T. Triterpene alcohol and fatty acid composition of shea nuts from seven African countries. *J. Oleo Sci.* **59**, 351-360 (2010).
- 14) Akihisa, T.; Tokuda, H.; Yasukawa, K.; Ukiya, M.; Kiyota, A.; Sakamoto, N.; Suzuki, T.; Tanabe, N.; Nishino, H. Azaphilones, furanoisophthalides, and amino acids from the extracts of *Monascus pilosus*-fermented rice (red-mold rice) and their chemopreventive effects. *J. Agric. Food Chem.* **53**, 562-565 (2005).
- 15) Akihisa, T.; Kojima, N.; Kikuchi, T.; Yasukawa, K.; Tokuda, H.; Masters, E. T.; Manosroi, A.; Manosroi, J. Anti-inflammatory and chemopreventive effects of triterpene cinnamates and acetates from shea fat. *J. Oleo Sci.* **59**, 351-360 (2010).
- 16) Siddiqui, B. S.; Afshan, F.; Gulzar, T.; Hanif, M. Tetracyclic triterpenoids from the leaves of *Azadirachta indica*. *Phytochem.* **65**, 2363-2367 (2004).
- 17) Kraus, W.; Cramer, R.; Sawitzki, G. Tetranortriterpenoids from the seeds of *Azadirachta indica*. *Phytochem.* **20**, 117-120 (1981).
- 18) Conformational analysis: Global minimum conformation search of compound 16 was done using CAChe CONFLEX with MM2 force field (CAChe version 5.5; Fujitsu Co., Tokyo, Japan).
- 19) Lim, Y.-J.; Lee, E. H.; Kang, T. H.; Ha, S. K.; Oh, M. S.; Kim, S. M.; Yoon, T.-J.; Kang, C.; Park, J.-H.; Kim, S. Y. Inhibitory effects of arbutin on melanin biosynthesis of α -melanocyte stimulating hormone-induced hyperpigmentation in cultured brownish guinea pig skin tissues. *Arch. Pharm. Res.* **32**, 367-373 (2009).
- 20) Akihisa, T.; Yasukawa, K.; Tokuda, H. Potentially cancer chemopreventive and anti-inflammatory terpenoids from natural sources. in *Studies in Natural Products Chemistry, Vol. 29, Bioactive Natural Products (Part J)* (Atta-ur-Rahman, ed.) Elsevier Science B. V. Amsterdam. pp 73-126 (2003).
- 21) Niles, R. M. Biomarker and animal models for assessment of retinoid efficacy in cancer chemoprevention. *Acta Pharmacol. Sin.* **28**, 1383-1391 (2007).
- 22) Momchilova, S.; Antonova, D.; Marekov, I.; Kuleva, L.; Nikolova-Damyanova, B. Fatty acids, triacylglycerols, and sterols in neem oil (*Azadirachta indica* A. Juss) as determined by a combination of chromatographic and spectral techniques. *J. Liquid Chromatogr. Rel. Technol.* **30**, 11-25 (2007).
- 23) Bhargava, K. P.; Gupta, M. B.; Gupta, G. P.; Mitra, C. R. Antiinflammatory activity of saponins and other natural products. *Indian J. Med. Res.* **58**, 724-730 (1970).
- 24) Okpanyi, S. N.; Ezeukwu, G. C. Anti-inflammatory and antipyretic activities of *Azadirachta indica*. *Planta Med.* **41**, 34-39 (1981).