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Limonoids from the fruits of *Melia azedarach* and their cytotoxic activities

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ABSTRACT

Thirty-one limonoids and one tirucallane-type triterpenoid were isolated from the fruits of *Melia azedarach* (Meliaceae). The structures of 14 of these isolated compounds were elucidated on the basis of spectroscopic analyses and comparison with literature. All of these compounds were evaluated for their cytotoxic activities against HL60, A549, AZ521, and SK-BR-3 human cancer cell lines. Meliarachin C (IC_{50} 0.65 μM) and 3-*O*-deacetyl-4′-demethyl-28-oxosalannin (IC_{50} 2.8 μM) exhibited potent cytotoxic activity against HL60 cells, and this was demonstrated mainly due to the induction of apoptosis by flow cytometry. Western blot analysis suggested that both compounds induced apoptosis *via* both the mitochondrial and death receptor-mediated pathways. In addition, 25 compounds were evaluated for their inhibitory effects against the Epstein–Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells.

1. Introduction

Plants of the Meliaceae family have been well documented for their ability to metabolize structurally diverse and biologically significant limonoids and triterpenoids (Tan and Luo, 2011; Zhao et al., 2010). In the course of a search for potential bioactive compounds from Meliaceae plants, a detailed investigation on the limonoid constituents of *Azadirachta indica* (neem) seed extracts was undertaken and this showed that some limonoids exhibit potent inhibitory activities 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 (Akihisa et al., 2009, 2011), as well as cytotoxic and apoptosis-inducing activities (Kikuchi et al., 2011). In a continuing study on the limonoid constituents of Meliaceae plants, the fruit extract of *Melia azedarach* L., was investigated, and thirty-one limonoids (**1**–**31**) were isolated, including 14 new compounds, and one tirucallane-type triterpenoid (**32**). This paper describes the structure elucidation of these compounds and evaluation of their cytotoxic activities and inhibitory effects on the induction of EBV-EA activation induced with TPA in Raji cells. *M. azedarach* is indigenous to Japan and other countries in the southeastern Asia. Its bark and fruit, which are known as "Kurenpi" and "Kurenshi", respectively, in Japan, have been used for vermicide, anodyne, and skin disease (Namba, 1994; Okada, 2002). Many constituents including limonoids, triterpenoids, and steroids have been isolated from various parts of *M. azedarach* (Carpinella et al., 2003; Liu et al., 2011; Nakatani et al., 1995; Ntalli et al., 2010; Ochi et al., 1978a; Su et al., 2011; Wu et al., 2011; Zhou et al., 2004, 2005). Several of the limonoids isolated from *M. azedarach* have been reported to possess antimicrobial (Liu et al., 2011; Su et al., 2011), cytotoxic (Wu et al., 2011; Zhou et al., 2004), antifeedant (Carpinella et al., 2003; Nakatani et al., 1995), and insecticidal (Carpinella et al., 2003) activities.

2. Results and discussion

2.1. Cytotoxic activity of the extracts of M. azedarach fruits

Dried and powdered *M. azedarach* fruits were extracted with *n*-hexane, and the defatted residue was then extracted with MeOH. The MeOH extract was fractionated into EtOAc-, *n*-BuOH-, and H_2O-soluble fractions. The *n*-hexane and MeOH extracts and the three fractions from the MeOH extract were evaluated for their cytotoxic activities against four human cancer cell lines: HL60 (leukemia), A549 (lung), AZ521 (stomach), and SK-BR-3 (breast), by means of a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay, and the results are summarized in Table 1. The EtOAc-soluble fraction exhibited potent activity against HL60, A549, and AZ521 cells, and the *n*-BuOH-soluble

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Table 1
Cytotoxicity of extracts and fractions of *Melia azedarach* fruits against four human cancer cell lines.

Extract and fraction	IC_{50} (ng/ml)[a]			
	HL 60 (Leukemia)	A549 (Lung)	AZ521 (Stomach)	SK-BR-3 (Breast)
n-Hexane extract	75.5 ± 1.7	>100	77.0 ± 1.1	>100
MeOH extract	2.9 ± 0.4	64.1 ± 5.4	2.9 ± 1.3	21.0 ± 3.3
EtOAc-soluble fraction	0.05[b] ± 0.006	4.2 ± 0.5	1.9 ± 0.2	34.7 ± 2.4
n-BuOH-soluble fraction	1.9 ± 0.4	>100	2.0 ± 0.3	>100
H_2O-soluble fraction	>100	>100	>100	>100

[a] IC_{50} Values based on quintuple points. Cells were treated with test samples (1×10^{-4}–1×10^{-6} g/ml) for 48 h, and cell viability was analyzed by the MTT assay. Each value represents the mean ± SD ($n = 3$).
[b] Range of concentration of test sample assayed: 1×10^{-6}–1×10^{-8} g/ml.

fraction against HL60 and AZ521 cells. The EtOAc-soluble fraction was further investigated for their constituents in this study.

2.2. Isolation, identification, and structure elucidation

The EtOAc-soluble fraction was subjected to successive column chromatography (CC) on silica gel (SiO_2) and octadecyl silica gel (ODS) columns, and to reversed-phase HPLC which led to the isolation of thirty-one limonoids, **1**–**31**, including 14 new compounds, **5**, **6**, **13**, **15**, and **17**–**26**, and one tirucallane-type triterpenoid, **32**. Among these compounds, 17 known compounds were identified as meliarachin C (**1**) (Su et al., 2011), toosendanin (**2**; C-29 epimeric mixture) (Kim et al., 1999; Ochi et al., 1978a,b), meliarachin K (**3**) (Su et al., 2011), meliarachin G (**4**) (Su et al., 2011), trichilinin D (**7**) (Nakatani et al., 1999), 1-*O*-cinnamoyltrichilinin (**8**) (Rajab and Bentley, 1988), salannin (**9**) (Johnson and Morgan, 1997), 3-*O*-deacetylsalannin (**10**) (Madyastha and Venkatakrishnan, 2000), 3-*O*-deacetyl-3-*O*-tigloylsalannin (**11**) (Madyastha and Venkatakrishnan, 2000), ohchinin (**12**) (Fukuyama et al., 1983), ohchinin acetate (**14**) (Ochi et al., 1978b), 1-*O*-decinnamoyl-1-*O*-benzoylohchinin acetate (**16**) (Ochi et al., 1978b), ohchinolal (**27**) (Fukuyama et al., 1983), mesendanin E (**28**) (Dong et al., 2010), 1-*O*-detigloyl-1-*O*-benzoylohchinolal (**29**) (Zhou et al., 2004), 1-*O*-detigloyl-1-*O*-cinnamoylohchinolal (**30**) (Zhou et al., 2004), nimbolinin D (**31**) (Nakatani et al., 2000), and meliasenin E (**32**) (Zhang et al., 2010) by MS, ^1H NMR, and ^{13}C NMR spectroscopic comparison with corresponding literature data (Fig. 1).

The structures of the 14 new compounds were elucidated on the basis of spectroscopic data and by comparison with literature as described below, and their proposed structures were supported by analysis of the DEPT, ^1H–^1H COSY, HMQC, HMBC, and NOESY data.

The molecular formula of compound **5** was determined as $C_{29}H_{38}O_9$ by HRESIMS. The ^1H and ^{13}C NMR spectroscopic data of **5** indicated that it was structurally similar to compound **3** (Su et al., 2011), with the only difference being the absence of an AcO group. The AcO group was located at C-3 of **5** by the HMBC for H-3 (δ_H 4.92) and the AcO group (δ_C 169.8). The NOESY correlation (Fig. 2) between H-3 and H-29 supported the *S*-configuration at C-29 (29-*endo*). Hence, the structure of compound **5** was assigned as (29*S*)-19,29-epoxy-29-*O*-methyl- 1α,3α,7α,29-tetrahydroxymeliacane-11,15-dione 3-acetate which was named 12-dehydroneoazedarachin D.

Compound **6** had the molecular formula $C_{29}H_{38}O_9$ as determined by HRESIMS. The ^1H and ^{13}C NMR spectra (Tables 2 and 5) of **6** resembled those of **5**, except for the downfield shift of H-3 signal (δ_H 5.35) suggesting that **6** is a 29-*exo*-isomer (Huang et al., 1994) of **5**. Thus, **6** was assigned as (29*R*)-19,29-epoxy-29-*O*-methyl-1α,3α,7α,29-tetrahydroxymeliacane-11,15-dione 3-acetate (12-dehydro-29-*exo*-neoazedarachin D). The NOESY correlation (Fig. 2) between H_2-19 and H-29 supported the configuration.

The HRESIMS of compound **13** displayed a quasi molecular ion peak at *m*/*z* 603.2945 ($[M+H]^+$) consistent with the molecular formula of $C_{36}H_{42}O_8$. The ^1H and ^{13}C NMR spectra of **13** were almost superimposable on those of **12** (Fukuyama et al., 1983), except for the coupling constant between the ^1H signals of H-2′ and H-3′. Compound **12** exhibited the resonances with larger coupling constant between these signals ($J_{H\text{-}2',3'}$ = 15.9 Hz) (Fukuyama et al., 1983), while compound **13** showed them with smaller coupling constant ($J_{H\text{-}2',3'}$ = 12.4 Hz) which implied that the latter was the *cis*-diastereoisomer of the former (Akihisa et al., 2000). Thus, structure **13** was assigned as 1-*O*-*Z*-cinnamoylsalannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-*Z*-cinnamoylohchinin). The *cis*-cinnamoyl group of **13** was supported from the NOE correlation between the ^1H signals of H-2′ and H-3′ (Fig. 2).

The HRESIMS of compound **15** displayed a sodiated molecular ion peak at *m*/*z* 599.2543 $[M+Na]^+$ corresponding to the molecular formula $C_{34}H_{40}O_8$. The ^1H and ^{13}C NMR spectroscopic data (Tables 2 and 5) of **15** were similar to those of **13**, except for the presence of a benzoyl group and the absence of a *cis*-cinnamoyl group. In HMBC experiments, the H-1 signal at δ_H 5.22 showed a cross-peak with the carbonyl resonance (δ_C 165.1) of the benzoyl group, indicating that **13** had a benzoyl group at the C-1 position in place of a *cis*-cinnamoyl group in **13**. Thus, structure **15** was elucidated as 1-*O*-benzoylsalannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-benzoylohchinin).

Compound **17** was shown to have the molecular formula $C_{36}H_{42}O_9$ by HRESIMS (*m*/*z* 641.2724 ($[M+Na]^+$, $C_{36}H_{42}O_9Na^+$). The ^1H and ^{13}C NMR spectroscopic data (Tables 2 and 5) of **17** were analogous to those of compound **12** (Fukuyama et al., 1983), although the β-furyl ring signals for **12** were lacking for **17**. The presence of an α,β-unsaturated-γ-lactone ring instead of the β-furyl ring at C-17 was deduced by the ^1H [δ_H 4.27 and 4.50 (each 1H; H_2-23), and 7.06 (H-22)] and ^{13}C [δ_C 135.1 (C-20), 174.3 (C-21), 145.0 (C-22), and 70.0 (C-23)] signals (Mohamad et al., 2009). The HMBC correlations for H-17 and the C-20, C-21, and C-22 indicated the presence of β-substituted-γ-lactone ring. Hence, the structure of **17** was assigned as 1-*O*-cinnamoyl-17-defurano-17-(2-buten-4-olide-2-yl)-salannic acid methyl ester which was named ohchininolide. The NOE correlation between H-17 and Me-18 supported that the γ-lactone ring at C-17 was α-oriented.

Compound **18** was shown to have the molecular formula $C_{34}H_{40}O_9$ by HRESIMS. The ^1H and ^{13}C NMR spectroscopic data (Tables 3 and 5) of **18** resembled those of **17**, except for the presence of a benzoyl group and the absence of a cinnamoyl group. In HMBC experiments, the H-1 signal at δ_H 5.18 showed a cross-peak with the carbonyl resonance (δ_C 164.9) of the benzoyl group, indicating that **18** had a benzoyl group at C-1 position in place of a cinnamoyl group in **17**. Hence, structure **18** was elucidated as 1-*O*-benzoyl-17-defurano-17-(2-buten-4-olide-2-yl)-salannic acid methyl ester (1-*O*-decinnamoyl-1-*O*-benzoylohchininolide).

Compound **19** has the molecular formula of $C_{37}H_{44}O_{10}$, as determined by the sodiated ion at *m*/*z* 671.2821 ($[M+Na]^+$) in HRESIMS.