Minor Sesquiterpenoid and Steroid Constitutes from Azadirachta indica A. Juss and Their Cytotoxic Activity

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Abstract:
Background Neem (Azadirachta indica A. Juss) is a plant belonging to the melaiceae family. It was used widely as herbal medicine in ancient India and Burma. It possesses a variety of chemical constitutes. Recent studies showed that some of major constitutes have remarkable anticancer activities. However, the minor constitutes in neem are still studied insufficiently.

Aim The aim of the study is to identify the minor constitutes in neem, and investigate their cytotoxic activity.

Material & methods The dry seeds of neem were extracted with 95% ethanol. The ethyl acetate fraction of the extract was systematically separated by silica gel, Sephadex LH-20, High Performance Liquid Chromatography (HPLC) and other chromatographic techniques. The structures of the resulting isolates were elucidated by spectroscopic methods, such as mass spectrum (MS), nuclear magnetic resonance spectrum (NMR), etc.

Results A sesquiterpenoid, 11,13-dihydroqinghaosu V (1), together with two known steroids, (24R)-ergosta-5,7,22E-trien-3β-ol (2) and 5α,8α-epidioxiergosta-6,22-dien-3β-ol (3), was isolated from the dry seeds of neem. 1 is a compound which is isolated from nature for the first time. In vitro cytotoxic bioassays showed that compound 1 selectively inhibited the growth of Hela cell lines, with an IC₅₀ value of 37.26 ±6.02 μM.

Conclusion The study indicated that besides some major constituents of neem possessing anticancer activity, its minor metabolites may also inhibit growth of cancer cells.

Key words Azadirachta indica, sesquiterpenoid, steroid, cytotoxic activity.

INTRODUCTION

Neem (Azadirachta indica A. Juss) belongs to Meliaceae family. It originally distributes in India and Burma, and has been widely planted in southeast Asia, Africa, America and Australia[1,2]. Neem was used as herbal medicine in India and Burma to treat wound infection, hypertension, chickenpox, ulcer, fever, skin rash and many other diseases[1,3,4]. Many compounds isolated from neem have been recently found to show remarkable anticancer
activity. However, all of these works only focus on its main constituents, such as nimbolide\(^5\)–\(^8\) and azadirone\(^9\), while its minor metabolites were little studied. Our systematic investigation on the chemical constituents of the extracts of neem seeds led to isolation of nineteen limonoids including six new ones\(^10\)–\(^13\). Among these compounds, azadiramine A\(^11\), 28-deoxo-2,3-dihyronimbolide\(^10\) and 28-deoxonimbolide10 showed inhibitory activity against distinct human cancer cells. In the present work, we reported isolation, structural elucidation and cytotoxic bioassays of one sesquiterpenoid and two steroids.

**MATERIALS AND METHODS**

**GENERAL**

NMR spectra were acquired on a Bruker Avance III-600 and a Bruker Avance III-300 instruments (Bruker, Bremerhaven, Germany). High-resolution mass spectra were obtained on a LCQ Advantage MAX (Finnign, USA). Silicagel (80–100 and200–300 mesh, Qingdao Haiyang, Qingdao, China), Sephadex LH-20(Pfamadex), and RP-C18 (AA12S50, YMC) were used for column chromatography. Preparative HPLC was carried out using an Ultimate 3000 instrument (Thermo Scientific, USA) with a Waters X Bridge RP-C18 column (250 mm× 10 mm). Analytical HPLC was run on an Agilent 1260 instrument (Agilent, USA) with a Phenomenex Synergi RP-C18 column (250mm× 4.6 mm).

**PLANT MATERIAL**

The seeds of Azadirachta indica A. Juss were collected in Yunnan province and authenticated by Prof. Hanhong Xu, College of Resource and Environmental Engineering, South China Agricultural University. A voucher specimen was deposited in the College of Pharmacy, Jinan University, P.R. China.

**ISOLATION**

The air-dried, powdered seeds of A. Indica (15.0 kg) were extracted by refluxing with 120.0 L of 95% EtOH twice. Then the combined EtOH extracts were concentrated under reduced pressure to give a crude residue (414.7 g). The residue was suspended in 2.5 L distilled H\(_2\)O and partitioned with petroleum ether (PE), EtOAc and n-BuOH (3 × 3.0 L for each solvent). After removal of the solvent under vacuum, the EtOAc (198.5 g) fraction was subjected to a silica gel column chromatography eluted with a gradient of increasing acetone (0–50%) in PE, followed by a gradient of increasing MeOH (5%–100%) in EtOAc, to afford 20 fractions (Fr.1–20). Fr.8 (3.6 g) was separated with silica gel again by a gradient of increasing acetone (15–30%) in PE, to obtain 18 sub-fractions (Fr.8a–Fr.8r). Fr.8f was purified by semi-preparative HPLC (4 mL/min, 70% MeOH in H\(_2\)O) to yield compounds 3 (13.6 mg, \(t_\beta\) 36.95 min). Fr.9 (1.8 g) was separated by Sephadex LH-20 to afford 9 sub-fractions (Fr.9a–Fr.9i), using PE: CH\(_2\)Cl\(_2\): MeOH (5:4:1) as mobile phase. Fr.9e (78.6 mg) was purified by semi-preparative HPLC (4 mL/min, 65% MeOH in H\(_2\)O) to yield compounds 2 (13.5 mg, \(t_\beta\) 42.65 min). Fr.14 (7.8 g) was chromatographed on an ODS column (RP-18, AA12S50, 200g) by eluting with a gradient of MeOH (55%–65%–75%–85%–100%) in H\(_2\)O to obtain 25 sub-fractions (Fr.14a–Fr.14y). Fr.14p (76.5 mg) was purified by reversed-phase semi-preparative HPLC (4 mL/min, 58%MeOH) to obtain compounds 1 (9.6 mg, \(t_\beta\) 26.35 min).

**CYTOTOXIC ASSAY**

Human breast cancer cell line (MCF-7), human liver carcinoma cell line (HepG2), human leukemia cell line (HL-60), and human cervix epithelioid carcinoma cell line (HeLa) were provided by Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China. The isolates were evaluated their cytotoxic effects against the above cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method, as described previously\(^14\). Cisplatin was used as the positive control. All cells were cultured in RPMI1640
medium supplemented with 10% FBS and antibiotics (Penicillin 100IU/mL, Streptomycin 100 μg/mL), and incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cisplatin was used as the positive control. The absorption at 570 nm was measured using a microplate reader (Perkin Elmer, 1420 Multilabel Counter Victor 3, Wellesley, MA, USA).

RESULTS

The dried and powered seeds of *Azadirachta indica* were extracted by refluxing with 95% ethanol. The EtOAc fraction of the total extract was separated using various chromatography methods, i.e. silica gel, SephadexLH-20, ODS and preparative HPLC to afford compounds 1-3 (Fig. 1).

![Compound structures](image)

Fig. 1 The structures of 1-3.

Compound 1 was obtained as white needle crystals. The molecular formula of 1 was established to be C_{15}H_{24}O_{3} with four degrees of unsaturation, based on a quasi-molecular ion peak at m/z 275.1584 [M + Na]⁺ in its HR-ESI-MS spectrum. The ¹H NMR spectrum of 1 (Table 1) showed a proton signal on an oxygen-bearing carbon at δ_H 4.32 (1H, d, J = 11.8 Hz, H-5), and three proton signals attributed to three methyl groups at δ_H 1.34 (3H, s, H-3-14), 1.21 (3H, d, J = 7.2 Hz, H-3-13) and 0.87 (3H, d, J = 6.6 Hz, H-3-15). The ¹³C NMR spectrum (Table 1) indicated compound 1 had 15 carbon atoms, including a carbonyl carbon with a signal at δ_C 175.1 (C-12) and two oxygen-bearing carbons with signals at δ_C 80.9 (C-5) and δ_C 70.7 (C-4).

<table>
<thead>
<tr>
<th>No.</th>
<th>δ_C (ppm)</th>
<th>δ_H (J in Hz)</th>
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<th>δ_C (ppm)</th>
<th>δ_H (J in Hz)</th>
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<td>1</td>
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<td>1.47 (m, 1H)</td>
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<td></td>
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<tr>
<td>3</td>
<td>32.6</td>
<td>1.66 (m, 1H)</td>
<td>11</td>
<td>40.4</td>
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<tr>
<td>4</td>
<td>70.7</td>
<td></td>
<td>12</td>
<td>175.1</td>
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<td>80.9</td>
<td>4.32 (d, J = 11.8 Hz, 1H)</td>
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<td>13.9</td>
<td>1.21 (d, J = 7.2 Hz, 3H)</td>
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<td>2.37 (td, J = 11.8 Hz, 1H)</td>
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<td>7</td>
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<td>1.89 (m, 1H)</td>
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<td>20.0</td>
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<td>8</td>
<td>22.9</td>
<td>1.88 (m, 1H)</td>
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The structure of 1 was finally constructed by its 2D NMR spectra (Fig. 2). The proton and protonated carbon resonances in the NMR spectra were firstly assigned by the HSQC
experiment. The $^1$H-$^1$H COSY correlations of H-1/H-6, H-5/H-6/H-7/H-8/H-9/H-10 and H7/H11/H13 indicated the presence of the fragment labeled in red in Fig. 2. In the HMBC spectrum, the correlations from H$_2$-13 to C-7, C-11 and C-12, from H$_3$-14 to C-3, C-4 and C-5, from H$_3$-15 to C-1, C-9 and C-10, and from H-2 to C-1, C-3, C-4, C-6 and C-10, enable all the carbon atoms being tethered together. To meet degree of unsaturation of 1, there must be the third ring in its structure by forming an ester bond between the carbonyl carbon (C-12) and either C-4 or C-5. Due to the chemical shift of C-5 ($\delta_C$ 80.9) and C-4 ($\delta_C$ 70.7), we readily inferred that the ester bond is formed between C-5 and C-12. Therefore, the planar structure of 1 was elucidated.

The relative configuration of 1 was determined by its NOESY spectrum (Fig. 2). The NOE correlations of H-5/H-3/H-6/H-13, H-6/H-10 and H-5/H-1 indicated these protons were oriented on the same side of the ring system and arbitrarily assigned as $\beta$. The correlations of H$_3$-15/H-7/H-11 suggested they oriented on the opposite side. Herein, compound 1 was determined as 11,13-dihydroqinghaosu V$^{[15]}$. It is isolated from the nature for the first time.

The cytotoxic activities of these isolated compounds were evaluated using MTT assay. The results showed that compound 1 selectively inhibit the growth of Hela cell lines, with IC$_{50}$ value of 37.26 ±6.02 µM. The IC$_{50}$ values of the positive control cisplatin against MCF-7, HepG2, HL-60 and HeLa cells were 11.43 ± 2.83, 2.46 ± 0.14, 1.70 ±0.43, 8.96± 1.59 µM, respectively. While compounds 2 and 3 didn’t shows significant inhibitory activity against all the cancer cell lines tested.

CONCLUSION
A sesquiterpenoid (1) and two steroids (2 and 3) were isolated from neem seeds. 1 is the compound which is found in nature for the first time and possesses selective cytotoxic activity against Hela cell lines. Our findings indicate that more natural products with anticancer activity could be found from minor constitutes of neem.

REFERENCES
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**Conflicts of Interest:** No conflict of interest.

**Author contribution:** Hua Dong and Xiaofeng Lu performed the experiments; Jiachen Zi and Xiaona Fan analyzed the data; Jiachen Zi and Xiaona Fan wrote the paper.

**Statement of originality of work:** The manuscript has been read and approved by all the authors, the requirements for authorship have been met, and that each author believes that the manuscript represents honest and original work.

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