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Pentacyclic Triterpenes from the Ethyl Acetate Fraction of the Bark of *Platanus acerifolia* Wild and Antitumor Activities *In Vitro*

Heng-Zhi Wang[1], Chuan-Jin Wang[2]* and Wei Li[2]

[1]*Nanjing No.1 Middle School, Nanjing 210028, China*
[2]*Department of Pharmaceutical Engineering, Institute of Chemical Engineering, Nanjing University of Science and Technology, Nanjing 210094, China*

Abstract

Three pentacyclic triterpenes, named betulinic acid **(1)**, 11α-hydroxy-β-amyrin **(2)** 3β-acetoxy-20 (29)-lupen-28-aldehyde **(3)** were isolated from the ethyl acetate fraction of the bark of *Platanus acerifolia* Willd. The molecular structure of **1** and were established on the basis of various spectroscopic analyses. The molecular structure of **(3)** was determined by single-crystal X-ray diffraction. Compound **(2)** and **(3)** were obtained from the title plant for the first time. Cytotoxicity of the isolated compounds against three human cancer cell lines, HepG-2, MCF-7 and HL-60 were also determined with the cell counting kit-8 (CCK-8) assay. The target compounds showed the high cytotoxicity, with IC_{50} values in the range 2.2-9.1 μM. These results indicated that pentacyclic triterpenes from the bark of *Platanus acerifolia* Willd could be explored as potential cancer prevention agents.

Keywords: Pentacyclic triterpenes; *Platanus acerifolia* Willd bark; Antitumor activity *in vitro*

Introduction

Platanus acerifolia Willd, one of the famous street and garden trees, is a very large, wide spreading, and long-lived hardwood species native to Eurasia [1]. The bark of *Platanus acerifolia* Willd has been used as the traditional Chinese medicine in the treatment of dysentery, diarrhea, toothache and tumor [2]. In order to find some bioactive compounds, the chemical constituents of *Platanus acerifolia* Willd bark were investigated and three compounds, named betulinic acid **(1)**, 11α-hydroxy-β-amyrin **(2)** and 3β-acetoxy-20 (29)-lupen-28-aldehyde **(3)** were isolated. The structures of the three compounds were identified by their physicochemical properties and spectral analysis. In addition, the isolated compounds were also evaluated for cytotoxic efficacy against HepG-2, MCF-7 and HL-60 cell lines *in vitro*.

Materials and Methods

General experimental procedures

Melting points were determined on RD-2 micromelting point apparatus and are uncorrected. The ^{1}HNMR (500 MHz) and ^{13}C NMR (500 MHz) spectra were recorded on a Bruker Avance Ⅲ-500 spectrometer and tetramethylsilane (TMS) was used as an internal standard. Silica gel (200-300 mesh for Column Chromatography (CC) and GF_{254} for TLC) was obtained from Qingdao Marine Chemical Company (Qingdao, China). Sephadex LH-20 was obtained from Amersham Biosciences (Uppsala, Sweden). Single-crystal structure of compound 2 was measured on an Enraf-Nonius CAD4 diffractometer etc.

Plant material

The bark of *Platanus acerifolia* Willd was collected in Nanjing County, Jiangsu Province, China, in January 2010.

Extraction and isolation

The dried barks of *Platanus acerifolia* Willd (100 g) were cut into small pieces and extracted with EtOAc (1 liter × 3). The solvent was removed by rotary evaporation and the yellow brown extract (4.0 g) was obtained. The EtOAc extract was subjected to silica gel chromatography using stepwise elution with petroleum ether-CH_3COCH_3 (100:0, 100:1, 100:2, 100:4, 100:8, 100:16, 100:32, 100:100, and 0:100) to afford 90 fractions (F1-F90). F51-F60 (A) was permeated through Sephadex LH-20 using a MeOH-CH_3Cl (1:1) system to give 10 subfractions A1-A10. Fractions A4-A6 were further purified with recystallization with CH_3Cl-CH_3COCH_3 (1:1) system to afford compound **(1)** (1000 mg); Fraction of F8 was further purified with recystallization with CH_3Cl-CH_3COCH_3-MeOH (1:1:1) system to afford compound **(2)** (10 mg); F11-F20 (B) were permeated through Sephadex LH-20 using a MeOH-CH_3Cl (1:1) system to give 12 subfractions B1-B12. Fractions B4-B7 were further purified with recystallization with EtOAc-MeOH (1:1) system to afford compound **(3)** (20 mg).

Compounds identification

Compound (1): Compound (**1**) was readily identified as betulinic acid by the analysis of their NMR spectra and by the comparison with the data reported in literature [3]. ^{1}H-NMR(500 MHz, DMSO) δ_H = 12.08 (1H, brs, H-28), 4.69 (1H, brs, H-29a), 4.56 (1H, brs, H-29b), 4.28 (1H, brs, H-3), 1.64 (3H, s, H-30), 0.93 (3H, s, H-23), 0.88 (3H, s, H-27), 0.87 (3H, s, H-26), 0.76 (3H, s, H-24), 0.68 (3H, s, H-25); ^{13}C-NMR (500 MHz, DMSO) δ_C = 180.0 (C-28), 150.3 (C-20), 109.5 (C-29), 76.8 (C-3), 55.4 (C-17), 54.9 (C-5), 55.4 (C-5), 49.9 (C-9), 48.5 (C-19), 46.6 (C-18), 42.0 (C-14), 40.2 (C-8), 38.5 (C-4), 38.2 (C-1), 37.5 (C-13), 36.7 (C-10), 36.4 (C-22), 33.9 (C-7), 31.7 (C-16), 30.1 (C-15), 29.2 (C-21), 28.1 (C-23), 27.1 (C-2), 25.1 (C-12), 20.4 (C-11), 18.9 (C-30), 17.9 (C-6), 15.9 (C-26), 15.8 (C-24, 25), 14.4 (C-27).

Compound (2): The acicular crystal of 11α-hydroxy-β-amyrin was recrystallized in the mixture solution of CH_3Cl- CH_3COCH_3-MeOH (1:1:1), and single crystal was obtained in constant temperature (25°C) on the basis of this. m.p: 234~236°C. Elemental Anal. Calcd. (%) for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38; O, 7.23. Found (%): C, 81.20; H, 11.48; O, 7.32. ^{1}H-NMR(500 MHz, $CDCl_3$) δ_H = 5.24 (1H, d, H-12), 4.21 (1H, dd, H-11), 3.22 (1H, dd, H-3), 2.06 (1H, m, H-18), 1.94 (2H, m, H-21), 1.23 (3H, s, H-23), 1.08 (3H, s, H-27), 1.02 (3H, s, H-25), 1.02 (3H, s, H-26),

***Corresponding author:** Chuan-Jin Wang, Department of Pharmaceutical Engineering, Institute of Chemical Engineering, Nanjing University of Science and Technology, Nanjing, 210094, China, Tel: +862584315514; E-mail: wangchuanjin@njust.edu.cn

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0.88 (3H, s, H-29), 0.88 (3H, s, H-30), 0.86 (3H, s, H-24), 0.81 (3H, s, H-28); ^{13}C-NMR (500 MHz, $CDCl_3$) δ_C = 147.1 (C-13), 120.7 (C-12), 78.7 (C-3), 67.6 (C-11), 55.8 (C-9), 55.2 (C-5), 46.9 (C-18), 46.6 (C-19), 45.6 (C-14), 42.8 (C-8), 40.7 (C-1), 39.0 (C-4), 38.8 (C-10), 37.0 (C-22), 34.6(C-21), 33.2 (C-29), 33.0 (C-7), 32.1 (C-17), 31.1 (C-20), 28.5 (C-23), 28.1 (C-28), 27.5 (C-2), 27.2 (C-15), 25.6 (C-16), 25.2 (C-27), 23.6 (C-30), 21.0 (C-6), 20.1 (C-26), 18.5 (C-25), 15.6 (C-24). Physical and spectra data of the title compound were almost identical with those reported in the literature [4].

Compound (3): Compound **(3)**, was obtained as colorless crystals. ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H = 9.68 (1H, s, H-28), 4.77 (1H, d, J = 1.85 Hz, H-29a), 4.64 (1H, d, J = 1.85 Hz, H-29b), 4.48 (1H, m, H-3), 2.86 (1H, m, H-19), 2.06 (3H, s, H-32), 1.70 (3H, s, H-30), 0.98 (3H, s, H-23), 0.93 (3H, s, H-27), 0.86 (3H, s, H-26), 0.85 (3H, s, H-24), 0.84 (3H, s, H-25); ^{13}C-NMR (500 MHz, $CDCl_3$) δ_C = 206.6 (C-31), 171.0 (C-28), 149.7 (C-20), 110.2 (C-29), 80.9 (C-3), 59.3 (C-17), 55.4 (C-5), 50.4 (C-9), 47.5 (C-19), 47.4 (C-18), 42.6 (C-14), 40.8 (C-8), 38.7 (C-4), 38.4 (C-1), 38.2 (C-13), 37.8 (C-10), 34.3 (C-22), 33.1 (C-7), 29.9 (C-16), 29.7 (C-15), 29.2 (C-21), 28.8 (C-23), 25.5 (C-2), 23.7 (C-12), 21.3 (C-32), 20.0 (C-11), 19.0 (C-30), 18.2 (C-5), 16.7 (C-25), 16.5. Its relative configuration was further established by X-ray crystallographic analysis [5].

Antitumoral cytotoxic assay

HepG-2, MCF-7 and HL-60 cell lines were provided by the Nanjing University of Traditional Chinese Medicine Immunization Center. All cell lines were cultured in multi-well plates at 37°C in a humidified atmosphere of 5% CO_2 with RPMI-1640 medium containing 10% fetal bovine serum with 50U/mL penicillin and 50 μg/mL streptomycin.

In vitro anti-cell proliferative effects of compound **(1)** and **(2)** 11α-hydroxy-β-amyrin were determined on HepG-2, MCF-7 and HL-60 cell lines using the CCK-8 assay. Briefly, cells were counted, transferred into 96 well microtiter plates, and incubated for 24 h prior to the addition of test compounds. Compounds were dissolved in DMSO and diluted in sterile media, as necessary, to obtain the appropriate concentration. Exponentially growing cells of HepG-2, MCF-7 and HL-60 were made into single cell suspensions with 0.25% trypsin, at a cell concentration of 1×10^5/mL. 90 μL cells (9×10^3) were seeded into each well of a 96-well plate. HepG-2, MCF-7 and HL-60 cells were incubated for 24 h before they were treated with compound **(1)** and **(2)** which were in a medium containing 0.1% DMSO, which showed no inhibitory effect on cell growth. This experiment was performed using 6 different final drug concentrations (3.125, 6.25, 12.5, 25, 50, 100 μM). To each well was added 10μL of the appropriate drug. Control cells were treated with an equal volume of serum-free RPMI 1640 containing 0.1% DMSO. After cells had been cultured for 24 h, 10 μL CCK-8 was added to each well. One hour later, the cell concentrations were recorded with an automated microplate reader at 450 nm. Each sample was assayed in triplicate, and each assay was repeated twice. Results are expressed as the concentration yielding 50% inhibition (IC_{50}).

$$\text{The inhibition rate (\%)} = \left[\frac{\left(A_{control} - A_{experiment}\right)}{A_{control}} \times 100\%\right]$$

Data were expressed as mean ± SD. One-way analysis of variances and Fisher's least significant difference was performed using SAS 8.13. Differences were significant at P<0.05.

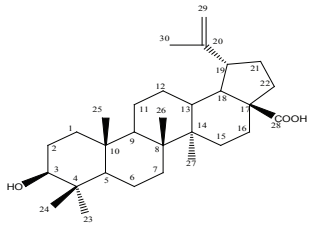
Results and Discussion

Compound **(1)** was readily identified as betulinic acid by the analysis of their NMR spectra and by the comparison with the data reported in literature (Figure 1) [3]. ^{1}H-NMR and ^{13}C-NMR spectra showed the typical pattern of pentacyclic triterpene. Especially, the ^{1}H-NMR spectrum of compound was characteristic of the presence of a vinyl protons at δ5.24 (1H, d) and 4.21 (1H, dd). Two C singlets at δ147.1 and 120.7 indicated the presence of C-C double bond. On the basis of the above evidences, compound **(2)** was suggested to 11α-hydroxy-β-amyrin. The NMR data of compound was in good agreement with the previous data of 11α-hydroxy-β-amyrin (Figure 1) [6]. Compound (**3**), was obtained as colorless crystals. The NMR data of compound (**3**) was in good agreement with the previous data of 3β-acetoxy-20 (29)-lupen-28-aldehyde [7]. Further single-crystal X-ray diffraction analysis confirmed the molecular structure of compound **(3)** (Figure 1) [4].

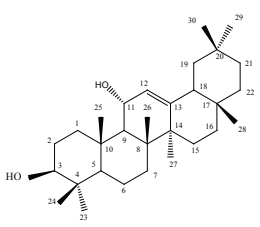
Cell Counting Kit-8 (CCK-8) is a reagent box used to detect cell proliferation, cell survival and cell toxicity based on a water soluble tetrazolium salt, WST-8{2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4–benzene disulfonate)-2H-

tetrazolium monosodium salt}. CCK-8 is an alternative to MTT assay. During the process of metabolism of living cells, in the presence of 1-methoxy PMS, the WST-8 in cells produces soluble orange formazon. The formazan generated is proportional to the number of living cells. Compared with MTT, CCK-8 has significant advantages. The formazan generated by MTT is not water-soluble, and requires specific solvents to dissolve it, such as dimethyl sulfoxide. However, formazan generated by CCK-8 solution is water-soluble, and thus organic solvents need not be used in the experiment. On the other hand, CCK-8 solution is fairly stable, not toxic to cells, and can be used directly [8].

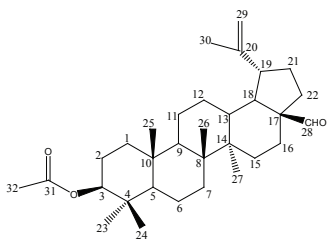
Compound **(1)**, **(2)** and **(3)** were evaluated for its cytotoxicity against HepG-2, MCF-7 and HL-60 cancer cell lines by using CCK-8 assays and Taxol as positive control (Table 1). Compound (**1**), **(2)** and **(3)** exhibited cytotoxicity against these cell lines and gave IC_{50} values in the range 2.2-9.1 μM. Two pentacyclic triterpenes from the ethyl acetate fraction of the bark of *Platanus acerifolia* Willd showed potent activities against the tested cancer cell lines. Compound **(3)** was 4.0 times more toxic to HepG-2 and MCF-7 cells than Compound (**1**),



Betulinic acid **(1)**



11α-hydroxy-β-amyrin **(2)**



3β-acetoxy-20 (29)-lupen-28-aldehyde **(3)**

Figure 1: Chemical structure of pentacyclic triterpenes.

Compounds	Growth inhibition constant (IC_{50})[a]		
	HepG-2	MCF-7	HL-60
Negative control	Nt	Nt	Nt
Solvent control	Nt	Nt	Nt
Compound (**1**)	9.1 ± 0.5 μM	8.7 ± 0.8 μM	6.1 ± 0.9 μM
Compound (**2**)	3.0 ± 0.8 μM	3.1 ± 0.5 μM	8.9 ± 1.5 μM
Compound (**3**)	2.3 ± 0.3 μM	2.2 ± 0.2 μM	5.3 ± 1.0 μM
Taxol[b]	1.8 ± 0.4 nM	5.1 nM	0.38 ± 0.2 nM

Table 1: Compound **(1)**, **(2)** and **(3)** against cultured HepG-2, MCF-7 and HL-60 cancer cell lines. [a]IC_{50} is defined as the concentration that resulted in a 50% decrease in cell number and the results are means ± standard deviation of three independent replicates.

indicating its potential as an anticancer drug. The cytotoxicity of these compounds against some cancer cell lines was previously reported [9-13].

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