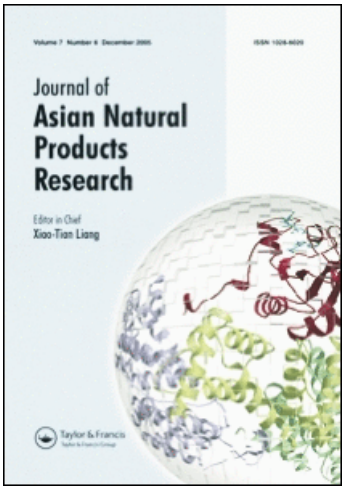

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Chemical constituents of the leaves of *Diospyros kaki* and their cytotoxic effects

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Chemical constituents of the leaves of *Diospyros kaki* and their cytotoxic effects

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Isolation and structure elucidation of two new compounds, kakispyrone (**1**) and kakisaponin A (**2**), together with 11 known compounds, from the leaves of *Diospyros kaki* L. are described. Their cytotoxic effects against several cancer cell lines (A549, HepG2 and HT29) are also reported.

Keywords: *Diospyros kaki* L; Kakispyrone; Kakisaponin A; Cytotoxic effect

1. Introduction

The genus *Diospyros* belongs to the family Ebenaceae and comprises about 500 species distributed in the tropical and temperate zone. Although many studies about quinone compounds in the *Diospyros* plants have been reported, there are few reports about constituents in leaves in spite of medicinal uses [1].

"Shi Ye" is the fresh or dry leaves of *Diospyros kaki* L. (Ebenaceae), which is widely distributed in East Asia. It is used in the treatment of hypertension, angina and internal haemorrhage in China [2], and has been used traditionally in Korea to promote maternal health [3]. Previous phytochemical studies on this plant revealed the presence of triterpenoids, flavonoids and phenolic compounds [1,4].

In this paper, we describe the isolation and structure elucidation of two new compounds, kakispyrone (**1**) and kakisaponin A (**2**), together with 11 known compounds, from the EtOAc extract of the leaves of *Diospyros kaki* L. collected in China. Among them, **3**, **4**, **5**, **6** were isolated from the *Diospyros* genus for the first time. The cytotoxic effects of these compounds against several cancer cell lines (A549, HepG2 and HT29) were also reported.

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2. Results and discussion

The EtOAc extract of the leaves of *Diospyros kaki* L. was fractionated by silica gel, Sephadex LH-20, polyamide, preparative TLC and preparative HPLC to obtain two new and 11 known compounds.

Compound **1** was obtained as yellow needles and gave a positive reaction with $FeCl_3$ reagent by TLC. Acid hydrolysis of **1** followed by TLC analysis of the hydrolysate and direct comparison with authentic sugars indicated the presence of D-glucose. The molecular formula of compound **1** was established as $C_{19}H_{20}O_9$ by HRESI-MS, which exhibited quasi-molecular ions at *m/z* 415.0998 $[M + Na]^+$ and 807.2104 $[2M + Na]^+$. The IR spectrum showed absorption bands of hydroxyl group (3392 cm^{-1}) and carbonyl group (1618 cm^{-1}) that were confirmed by ^{13}C NMR spectrum. The ^1H NMR spectrum of compound **1** presented phenol protons around δ 10.0, three aromatic signals at δ 7.65 (2H, brd, $J = 7.5$ Hz), 7.53 (1H, brt, $J = 7.0$ Hz) and 7.43 (2H, dd, $J = 7.5$, 7.0 Hz) indicating a monosubstituted benzene and two aromatic signals at δ 6.04 (1H, brs), and 5.98 (1H, brs). The ^{13}C NMR spectrum of **1** indicated a benzophenone derivative structure [5]. An anomeric proton signal was identified at δ 4.70 (1H, d, $J = 7.7$ Hz) by HMQC analysis. Its J value (>7 Hz) indicated the β-orientation at the anomeric centre of D-glucose. In the HMBC analysis, correlations were observed between anomeric proton with δ_C 157.5, and δ_H 6.04 with δ_C 157.5, 107.6, 162.8, 97.0, δ_H 5.98 with δ_C 94.8, 107.6, 162.8, 158.6, which placed the glucose moiety at C-2 position.

Further assignment of the proton and carbon signals was carried out by HMQC and HMBC correlations. From the above evidence, compound **1** was assigned as 4,6-dihydroxy-2-*O*-β-D-glucopyranosylbenzophenone and named kakispyrone, its structure and key HMBC correlations are shown in figure 1.

Compound **2** was obtained as colourless needles and gave a positive Lieberman–Burchard test for triterpenoids. Its IR spectrum showed absorption bands of hydroxyl group (3401 cm^{-1}) and carbonyl group (1721 cm^{-1}). The HRESI-MS of **2** showed quasi-molecular ions $[M + Na]^+$ and $[2M + Na]^+$ at *m/z* 673.3920 and 1323.7951, consistent with a molecular formula of $C_{36}H_{58}O_{10}$. The ^1H NMR and ^{13}C NMR spectra suggested the presence of a triterpene moiety and one sugar, clearly indicated by one anomeric proton (δ 6.28) and one anomeric carbon (δ 95.9), which was assigned by HMQC and HMBC correlations. Upon acid hydrolysis, compound **2** afforded barbinervic acid, identified by comparison of the ^{13}C NMR data with literature value [6], and D-glucose. Glycosidation shift could be observed in C-28 (δ 177.0 and 180.0) position by comparing its ^{13}C NMR data with those of the aglycone (barbinervic acid), and the correlation between anomeric proton and the C-28 (δ 177.0) was also observed in HMBC experiment, from which the location of glucose can be assigned. The further assignment was established by DEPT, HMQC and HMBC spectra. On the basis of these results, the structure of compound **2** named kakisaponin A, is proposed as shown in figure 2.

11 known compounds, 4,4′-dihydroxy-α-truxillic acid (**3**) [7], tatarine C (**4**) [8], myricetin (**5**) [9], annulatin (**6**) [9], trifolin (**7**) [9], astragalin (**8**) [9], hyperin (**9**) [9], isoquercetin (**10**) [9], rutin (**11**) [9], quercetin (**12**) [9], kampferol (**13**) [9], were also isolated and were identified by comparison of their spectral data and TLC behaviours with those of the authentic samples and reported spectroscopic data.

Thirteen isolated compounds were evaluated for their cytotoxicity against A549, HepG2, and HT29 cancer cell lines by using MTT assay and cyclophosphamide as positive control

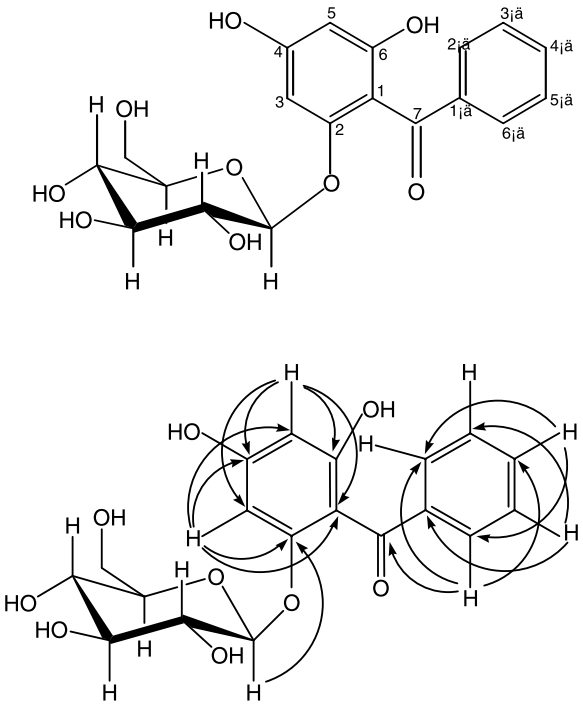

Figure 1. Structure and key HMBC correlations of **1**.

(table 3). Among them, compounds **2**, **3**, **4** exhibited cytotoxicity against these cell lines and gave IC_{50} values in the range 9.3–21.1 μM, while other compounds displayed no cytotoxicity effects against these cancer cell lines (>50 μM). Compound **2** showed significant cytotoxic efficacy against A549 cancer cell line. Moreover, it showed stronger cytotoxicity effect than its aglycone, barbinervic acid (data not shown), which suggested the relationship between the C-28 glucose and cytotoxicity activity.

3. Experimental

3.1 General experimental procedures

Melting points were measured on Yanaco micro-hot-stage apparatus and are uncorrected. NMR spectra were recorded on a Bruker-ARX-400 spectrometer. HRESI-MS was taken on a Bruker APEX II FT-ICRMS spectrometer. EI-MS was obtained on a VG7070E spectrometer. Bruker IFS 55 performed the IR spectrum. The chromatographic silica gel (200–300 mesh) and polyamide (100–140 mesh) were produced by Qingdao Ocean Chemical Factory; Sephadex LH-20 was purchased from Amersham Pharmacia Biotech. TLC analysis was performed on silica gel 60 F_{254} (Merck). All other chemicals and solvents used in this study were of reagent grade.

3.2 Plant material

The leaves of *Diospyros kaki* were collected in Xingtai, Hebei province of China, in September 2001. The plant was identified by Xu Chunquan, Professor of the Department of

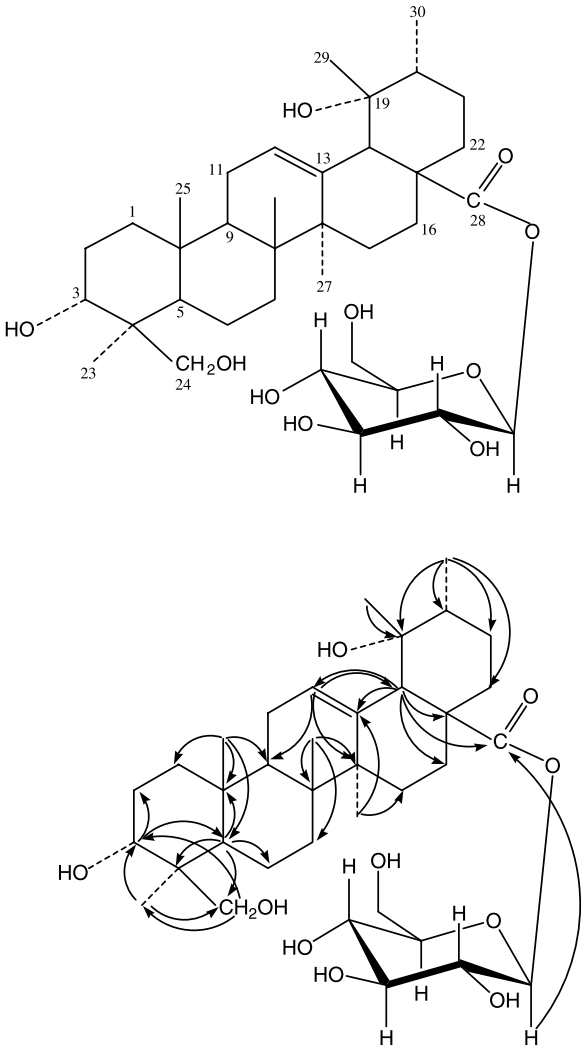

Figure 2. Structure and key HMBC correlations of **2**.

Natural Medicines, Shenyang Pharmaceutical University, and a voucher specimen has been deposited in the Herbarium of the Department of Natural Medicines, Shenyang Pharmaceutical University, Shenyang.

3.3 Extraction and isolation

Dried leaves of *Diospyros kaki* L. (7 kg) were extracted with 70% EtOH under reflux. After removal of solvent by evaporation, the combined extracts (1200 g) were suspended in H_2O and partitioned with petroleum ether. The aqueous layer was partitioned again with $CHCl_3$, EtOAC and n-BuOH successively. The EtOAC extract was silica gel chromatographed using mixtures of $CHCl_3$ and MeOH (50:1, 30:1, 10:1, 3:1, 1:1) as eluent, to obtain fractions A–G.

Fraction A was subjected to polyamide chromatography eluting with $CHCl_3$/MeOH (9:1) to give quercetin (**12**) (8.1 mg), kampferol (**13**) (7.6 mg) and myricetin (**5**) (8.7 mg).

Table 1. NMR data of compound **1** in DMSO-d_6.

Position	δ_H	δ_C
C-1	–	107.6
C-2	–	157.5
C-3	6.04 (1H, brs)	94.8
C-4	–	162.8
C-5	5.98 (1H, brs)	97.0
C-6	–	158.6
C-7	–	194.9
C-1′	–	139.5
C-2′, 6′	7.65 (2H, brd, $J = 7.5$ Hz)	128.8
C-4′	7.53 (1H, brt, $J = 7.0$ Hz)	132.0
C-3′, 5′	7.43 (2H, dd, $J = 7.5$, 7.0 Hz)	128.1
C-1″	4.70 (1H, d, $J = 7.7$ Hz)	100.6
C-2″	2.75 (1H, dd, $J = 8.1$, 7.7 Hz)	73.2
C-3″	3.16 (1H, m)	77.1
C-4″	3.03 (1H, dd, $J = 9.1$ Hz)	69.5
C-5″	3.13 (1H, m)	76.6
C-6″	3.64 (1H, brd, $J = 11.4$ Hz) 3.44 (1H, dd, $J = 11.4$, 4.7 Hz)	60.7

Fraction C was subjected to Sephadex LH-20 chromatography eluting with MeOH/H_2O (1:1) to give fractions 4–10. Fraction 4 was further chromatographed on Sephadex LH-20 eluting with MeOH/H_2O (2:1) to afford 4,4′-dihydroxy-α-truxillic acid (**3**) (20.3 mg) and annulatin (**6**) (9.8 mg); fraction 6 was purified by preparative TLC with $CHCl_3$/MeOH (6:1) to give tatarine C (**4**) (11.3 mg).

Fraction D was first subjected to Sephadex LH-20 chromatography eluting with MeOH/H_2O (1:1) to give fractions 1–3. Fraction 2 was further chromatographed on a silica gel column eluting with $CHCl_3$/MeOH (5:1) to give kakispyrone (**1**) (18.9 mg).

Fraction E was also subjected to polyamide chromatography eluting with $CHCl_3$/MeOH (8:1) to give fractions 1–3. Fractions 1 and 3 was subjected to Sephadex LH-20 to obtain trifolin (**7**) (8.9 mg), astragalin (**8**) (15.1 mg), hyperin (**9**) (11.0 mg) and isoquercetin (**10**) (12.5 mg). Fraction 2 was subjected to HPLC, developed with MeOH/H_2O (40:60), to afford kakisaponin A (**2**) (31.2 mg).

Fraction G was subjected to Sephadex LH-20 eluting with MeOH/H_2O (1:1) to give rutin (**11**) (21.6 mg).

3.3.1 Compound 1. Yellow needles (18.9 mg), mp 285–287°C. IR (KBr) λ_{max} (cm^{-1}): 3392, 1618, 1514, 1462; HRESI-MS: *m/z* 415.0998 $[M + Na]^+$, 807.2104 $[2M + Na]^+$ (calcd for $C_{19}H_{20}O_9Na$, 415.1005); ^1H NMR (DMSO-d_6, 400 MHz), ^{13}C NMR (DMSO-d_6, 100 MHz) data: see table 1.

3.3.2 Compound 2. Colourless needles (31.2 mg), mp 237–239°C. IR (KBr) λ_{max} (cm^{-1}): 3401, 1721; HRESI-MS: *m/z* 673.3920 $[M + Na]^+$, 1323.7951 $[2M + Na]^+$ (calcd for $C_{36}H_{58}O_{10}Na$, 673.3929); ^1H NMR (pyridine-d_5, 400 MHz), ^{13}C NMR (pyridine-d_5, 100 MHz) data: see table 2.

3.3.3 Compounds 3–13. Their ^1H NMR and ^{13}C NMR data were identical with those published in the literature.

Table 2. NMR data of compound **2** in pyridine-d_5.

Position	δ_H	δ_C
C-1	1.47 (2H, m)	34.0
C-2	1.82 (2H, m)	26.1
C-3	4.37 (1H, m)	70.0
C-4	–	43.9
C-5	1.90 (1H, brd, $J = 12.1$ Hz)	50.2
C-6	1.65 (2H, m)	19.3
C-7	2.05 (2H, m)	34.1
C-8	–	40.8
C-9	2.01 (1H, m)	47.9
C-10	–	37.5
C-11	2.02 (2H, m)	24.4
C-12	5.55 (1H, brs)	128.5
C-13	–	139.3
C-14	–	42.2
C-15	2.46 (2H, m)	29.2
C-16	1.82 (2H, m)	26.5
C-17	–	48.7
C-18	2.91 (1H, s)	54.5
C-19	–	72.7
C-20	1.33 (1H, m)	42.2
C-21	1.26 (2H, m)	26.7
C-22	2.04 (2H, m)	37.5
C-23	1.58 (3H, s)	23.6
C-24	3.82 (1H, d, $J = 10.8$ Hz)	65.8
	4.08 (1H, d, $J = 10.8$ Hz)	
C-25	1.02 (3H, s)	16.1
C-26	1.22 (3H, s)	17.4
C-27	1.60 (3H, s)	24.5
C-28	–	177.0
C-29	1.36 (3H, s)	27.0
C-30	1.04 (3H, d, $J = 6.1$ Hz)	16.7
C-1′	6.28 (1H, d, $J = 8.1$ Hz)	95.9
C-2′	4.19 (1H, m)	74.1
C-3′	4.21 (1H, m)	79.0
C-4′	4.29 (1H, m)	71.3
C-5′	4.02 (1H, m)	79.3
C-6′	4.34 (1H, d, $J = 9.2$ Hz)	62.4
	4.43 (1H, d, $J = 9.2$ Hz)	

3.4 Cell cultures

The human pulmonary adenocarcinoma cell line A549 and hepatic carcinoma cell line HepG2 were cultured in DMEM medium (high glucose), pH 7.3. Colorectal carcinoma cell line HT29 was cultured in PRIM-1640 medium, pH 7.3. Both cell cultures were

Table 3. Cytotoxicity effect of compounds **2**–**4** against A549, HepG2 and HT29 cancer cell lines.

	Growth inhibition constant (IC_{50})[†] (μM)		
Compounds	*A549*	*HepG2*	*HT29*
Cyclophosphamide[‡]	2.5 ± 1.8	2.7 ± 2.1	4.1 ± 1.6
2	9.3 ± 2.6	13.2 ± 2.3	18.1 ± 1.9
3	15.6 ± 3.1	16.1 ± 1.7	21.1 ± 2.3
4	10.1 ± 3.6	19.3 ± 1.9	17.4 ± 2.9

[†] 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.
[‡] Positive control substance.

supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% foetal calf serum (FCS). Cells were grown at 37°C under 5% CO_2 atmosphere. Culture media were changed every 2–3 days. When they reached confluence, cells were dissociated by 0.05% trypsin/0.02% EDTA and re-plated at 1:5 dilutions.

3.5 MTT assay

Cancer cells were seeded onto 96-well microtiter plates at 6×10^3 cells per well, and were pre-incubated for 24 h at 37°C. The medium was replaced with 180 μl fresh medium containing different concentrations of each isolated compound. The cells were then incubated at 37°C for 48 h, and then 20 μl of MTT was added to each well. After incubation in 37°C for 4 h, the supermatants were removed and the formazan crystals were dissolved by adding 200 μl DMSO. The plate was then read on a microplate reader at 490 nm to evaluate the effects of the test compounds on cell growth. Experiments were conducted in triplicate.

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