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Cytotoxic triterpenes from *Diospyros kaki* L. cv. costata (Ebenaceae)

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ABSTRACT
Four flavonoids (kaempferol-3-O-*β*-D-glucoside, quercetin-3-O-*β*-D-glucoside, kaempferol, and quercetin) and three triterpenes (ursolic acid, 3,19,24-trihydroxyurs-12-en and 3,19,24-trihydroxy-15-oxo-urs-12-en) were isolated from the ethyl acetate fraction of the alcoholic extract of *Diospyros kaki* L. cv. costata leaves cultivated in Egypt. The cytotoxic activity of 3,19,24-trihydroxyurs-12-en and 3,19,24-trihydroxy-15-oxo-urs-12-en was assessed on three human cell lines, cervical carcinoma (HELA), breast carcinoma (MCF7) and colon carcinoma (HCT116) cell lines using sulforhodamine-B assay. The two compounds exerted a moderate cytotoxic activity against the three tested cell lines comparing to doxorubicin as reference standard.

Keywords: *Diospyros kaki* L. cv. costata, cytotoxic, flavonoids, triterpenes

1. Introduction
A significant study of drug discovery in the last years has been focused on agents that prevent or treat cancer [1, 2]. Natural compounds from flowering plants have been playing an important role in the development of several clinically useful anticancer agents [3]. *Diospyros kaki* L. (Japanese persimmon) is a deciduous tree endogenous to Japan measuring to 12 m in heights and 7 m in diameters [4]. Its leaves are used as a traditional medicine for treatment of hypertension, angina and internal hemorrhage [5]. The leaves extract showed inhibitory effect on certain enzymes [6, 7] as well as it has antioxidant [8] and cytotoxic [9] activities. Triterpenes [7, 9, 13] and flavonoids [9] are the main constituents isolated from the leaves. In this study, the cytotoxic activity of two triterpenes, among the compounds isolated from the ethyl acetate fraction of the alcoholic extract of the leaves of Diospyros kaki L. cv. costata cultivated in Egypt, were evaluated on three human cell lines, cervical carcinoma (HELA), breast carcinoma (MCF7) and colon carcinoma (HCT116) cell lines.

2. Materials and Methods
2.1 General Experimental
Beckman Du-7 and Shimadzu-265 spectrophotometers were used for the determination of ultraviolet absorption spectra. Mass spectrometer, Varian Mat 711 (USA), Finnigan SSQ 7000 was used for EI/MS. 1H-(300 MHz) and 13C-(75 MHz) NMR spectra were recorded on Varian Mercury apparatus at 25 °C using TMS as an internal standard and chemical shifts were given in δ values. TLC was performed on precoated silica gel plates using suitable solvent systems, S_1 [EtOAC: MeOH: H_2O (5: 0.8: 0.6 v/v/v) and S_2 [$CHCl_3$: MeOH (95:5 v/v)]. The chromatograms were visualized under UV light (at λmax 254 and 366 nm) before and after exposure to ammonia vapor, as well as, spraying with p-anisaldehyde/sulphuric acid spray reagent. Reference samples for TLC comparison were obtained from E. Merck, Darmstadt, Germany.

2.2 Plant Material
Plant material of *Diospyros kaki* L. cv. costata were collected from El-Kanater El-Kireia, El-Kaliobia, Egypt. The plant was kindly identified by late ΦProf. Dr. El-Hadidi, Prof. of Plant Taxonomy, Botany Department, Faculty of Science, Cairo University. A voucher specimen (24-1-2010) was kept in the herbarium of Department of Pharmacognosy, Faculty of Pharmacy, Cairo University.

Φ May God bless his Soul

2.3 Extraction, Fractionation and Isolation:
The air dried, powdered leaves (450 g) of *Diospyros kaki* L. cv. costata was macerated in 70% ethyl alcohol till exhaustion. The combined ethanol extracts were evaporated under reduced pressure at a temperature not exceeding 60 °C to yield 75 g dry extract. The residue obtained was suspended in distilled water (500 ml) and successively extracted with petroleum ether (8 x 500 ml), chloroform (3 x 500 ml), ethyl acetate (6 x 500 ml) and *n*-butanol (4 x 500 ml). The solvent in each case was evaporated under reduced pressure to yield 18.5 g, 2.5 g, 7 g and 9.8 g, respectively.
Ethyl acetate fraction (7 g) was fractionated on a column of sephadex starting with CH_3OH: H_2O (1: 1) then increasing the amount of CH_3OH till 100% CH_3OH. Fractions (100 ml each fraction) were collected to obtain 38 fractions. The obtained fractions were monitored with TLC, similar fractions were pooled and the solvent was evaporated under reduced pressure. Fractions (8-10, 1.2 g) were chromatographed on a silica gel column using CH_3Cl: CH_3OH (97: 3) as a solvent system to obtain compounds 1 (60 mg), 2 (15 mg) and 3 (20 mg).

Fraction (24, 0.7 g) was chromatographed on sephadex using CH_3OH: H_2O (1: 1) as a solvent system to obtain compounds 4 (32 mg) and 5 (24 mg).
Fraction (38, 0.8) upon rechromatography on sephadex using CH_3OH as a solvent system afforded compounds 6 (100 mg) and 7 (54 mg).

Compound 1: white powder, soluble in chloroform, R_f = 0.68 in S_2, MS m/z: 456 $[M]^{+1}$.

Compound 2: white powder, soluble in chloroform, R_f = 0.45 in S_2, MS m/z: 458 $[M]^{+1}$. ^{1}HNMR (DMSO), δ ppm: 5.15 (1H, s, H-12), 3.73 (1H, br. s, H-3), 3.39 & 3.19 (2H, each d, *J*=9.6 & 10.8 Hz, H-24), 1.29 (3H, s, H-29), 1.27 (3H, s, H-27), 1.07 (3H, d, s, H-23), 0.88 (3H, s, H-28), 0.85 (3H, d, *J*=6.9 Hz, H-30), 0.82 (3H, s, H-26), 0.67 (3H, s, H-25). ^{13}C NMR (DMSO), δ ppm: 138.46 (C-13), 120.73 (C-12), 71.60 (C-19), 64.10 (C-3), 62.70 (C-24), 53.16 (C-18), 48.84 (C-5), 46.56 (C-19), 42.39 (C-4), 41.33 (C-20), 41.04 (C-14), 39.93 (C-8), 36.94 (C-22), 36.40 (C-10), 32.99 (C-7), 32.81 (C-1), 31.86 (C-17), 28.92 (C-15), 26.37 (C-29), 25.12 (C-21), 24.93 (C-2), 24.72 (C-16), 24.49 (C-27), 23.94 (C-11), 23.20 (C-23), 22.58 (C-28), 18.13 (C-6), 16.43 (C-26), 16.22 (C-30), 15.26 (C-25).

Compound 3: white powder, soluble in chloroform, R_f = 0.33 in S_2, MS m/z: 472 $[M]^{+1}$. ^{1}HNMR (DMSO), δ ppm: 5.15 (1H, br. s, H-12), 3.81 & 3.39 (2H, each d, *J*=10.8 Hz, H-24), 3.70 (1H, br. s, H-3), 1.27 (3H, s, H-29), 1.23 (3H, s, H-27), 1.07 (3H, d, s, H-23), 0.85 (9H, br. s, H-30, H-28 & H-26), 0.67 (3H, s, H-25). ^{13}C NMR (DMSO), δ ppm: 205.35 (C-15), 133.66 (C-13), 120.77 (C-12), 71.67 (C-19), 64.43 (C-3), 62.67 (C-24), 53.68 (C-18), 49.39 (C-5), 47.93 (C-14), 47.39 (C-17), 47.11 (C-19), 42.10 (C-4), 41.10 (C-20), 39.98 (C-8), 38.66 (C-22), 36.95 (C-10), 36.39, 33.59 (C-7), 33.36 (C-1), 26.91 (C-29), 26.43 (C-21), 25.67 (C-2), 25.67 (C-16), 24.49 (C-27), 23.75 (C-11), 22.91 (C-23), 17.35(C-6), 16.95 (C-26), 16.77 (C-30), 15.43 (C-25).

Compound 4: yellow powder, soluble in methanol, showed purple color in UV, no color with *p*-anisaldehy2de, R_f = 0.56 in S_1, UV λ_{max} nm: MeOH (266-348), $NaOCH_3$ (274-399), $AlCl_3$ (274-348 & 396), $AlCl_3$ / HCl (274-346 & 394), NaOAc (274-381), NaOAc / Boric acid (266-353), ^{1}HNMR (DMSO), δ ppm: 8.03 (2H, d, *J*= 7.2 Hz, H-2` & H-6`), 6.88 (2H, d, *J*=6.9 Hz, H-3`& H-5`), 6.44 (1H, d, *J*=1.8 Hz, H-8), 6.21 (1H, d, *J*=1.8 Hz, H-6), 5.44 (1H, d, *J*= Hz, H-1``), 3.10-3.85 (6H, m, sugar protons).

Compound 5: yellow powder, soluble in methanol, showed purple color in UV, yellow color with *p*-anisaldehyde, R_f = 0.54 in S_1, UV λ_{max} nm: MeOH (257-355), $NaOCH_3$ (272-405), $AlCl_3$ (274-428), $AlCl_3$ / HCl (269-358 & 399), NaOAc (272-380), NaOAc / Boric acid (262-375), ^{1}HNMR (DMSO), δ ppm: 7.52 (2H, m, H-2` & H-6`), 6.82 (1H, d, *J*= 8.4 Hz, H-5`), 6.39 (1H, d, *J*=1.8 Hz, H-8), 6.18 (1H, d, *J*=1.8 Hz, H-6), 5.42 (1H, d, *J*=6.6 Hz, H-1``), 3.07-3.74 (6H, m, sugar protons).

Compound 6: yellow powder, soluble in chloroform, R_f = 0.40 in S_4, UV λ_{max} nm: MeOH (266-366), $NaOCH_3$ (281-434), $AlCl_3$ (267-423), $AlCl_3$ / HCl (269- 4231), NaOAc (274-390), NaOAc / Boric acid (269-378), ^{1}HNMR (CD_3OD), δ ppm: 8.02 (2H, d, *J*= 8.7 Hz, H-2` & H-6`), 6.90 (2H, d, *J*=8.7 Hz, H-3`& H-5`), 6.43 (1H, d, *J*=1.8 Hz, H-8), 6.18 (1H, d, *J*=1.8 Hz, H-6).

Compound 7: yellow powder, soluble in chloroform, R_f = 0.36 in S_3, UV λ_{max} nm: MeOH (255-370), $NaOCH_3$ (331-429), $AlCl_3$ (270-425), $AlCl_3$ / HCl (267-429), NaOAc (269-399), NaOAc / Boric acid (260-387), ^{1}HNMR (DMSO), δ ppm: 7.65 (1H, d, *J*= 2.4 Hz, H-2`), 7.51 (1H, dd, *J*= 8.4 & 2.1 Hz, H-6`), 6.86 (1H, d, *J*=8.7 Hz, H-5`), 6.40 (1H, d, *J*=1.8 Hz, H-8), 6.18 (1H, d, *J*=1.8 Hz, H-6).

2.4 Cytotoxicity Study
Cervical carcinoma cell line (HELA), breast carcinoma cell line (MCF7) and colon carcinoma cell line (HCT116) were obtained from National Cancer Institute, Kasr El Ainy, Cairo, Egypt. Cytotoxic activity was tested using Sulforhodamine-B assay adopting the method of Skehan, 1990 **[14]**. Each assay was done in triplicate and the activity was expressed as IC_{50} which stands for inhibition of cancer cells growth by 50 %. The results obtained were compared with those of doxorubicin as reference standard (Table 1).

3. Results and Discussion:
Compound **1** was identified as ursolic acid depend on its MS and by TLC comparison with authentic sample. ^{1}H-NMR spectrum of compound **2** displayed resonances for six tertiary methyl (δ_H 1.29, 1.27, 1.07, 0.88, 0.82 and 0.67), a secondary methyl (δ_H 0.85), an oxygenated methine proton (δ_H 3.73), two exomethylene protons (δ_H 3.39 and 3.19) and an olefinic proton (δ_H 5.15). ^{13}C-NMR spectrum displayed signals of 30 carbons including an oxygenated quaternary carbon (δ_C 71.60). By comparing these data with the published data **[7, 10, 15, 16]** compound **2** was identified as 3,19,24-trihydroxyurs-12-en. NMR spectral data of compound **3** were closely similar to those of compound **2** with the presence of an additional signal for a carbonyl carbon at δ_C 205.35. The downfield shift of C-13 (δ_C 133.66) indicated the presence of the oxo group at C-15, thus compound 3 was identified as 3,19,24-trihydroxy-15-oxo-urs-12-en. The identification was confirmed by comparison with the published data **[16]**. Compound **2** and **3**

were isolated for the first time from *Diospyros kaki*.

Compounds **4-7** were identified based on their UV and ^{1}H-NMR spectral data and by comparison with the published data [17, 18] as kampferol-3-O-*β*-D-glucoside, quercetin-3-O-*β*-D-glucoside, kaempferol, and quercetin, respectively. The identification of compounds **4** and **5** was confirmed by acid hydrolysis [18] and comparison with authentic samples. Compounds **2** and **3** were tested for their cytotoxic activity against cervical carcinoma, breast carcinoma and colon carcinoma cell lines. From the results given in table 1, it could be concluded that both the two compounds exhibited moderate cytotoxic activity against the tested cell lines comparing to doxorubicin. Compound **3** recorded lower IC_{50} (higher potency) than compound **2** against colon carcinoma cell line (HCT116). This could be attributed to the presence of 15-oxo group in compound **3**. Cytotoxic activity for other triterpenes isolated from *Diospyros kaki* was previously reported [9].

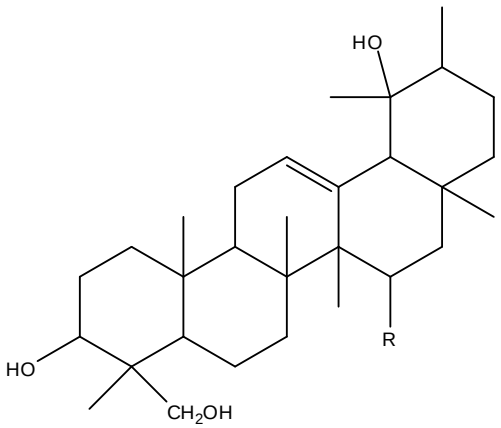

Fig 1: Structure of compounds 2 and 3

2: R H

3: R =O

Table 1: Cytotoxic activity of compounds 2 and 3

Cell line	IC_{50} (μg ml^{-1})		
	Compound 2	**Compound 3**	**Doxorubicin**
HELA	16.33 ± 2.08	15.83 ± 0.416	0.91 ± 0.1
MCF7	13.42 ± 2.26	14.93 ± 2.29	2.97 ± 0.05
HCT116	19.60 ± 0.69	14.33 ± 0.29	3.73 ± 0.21

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