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Phytoconstituents with cytotoxic activity from *Ulmus pumila* L.

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ABSTRACT

The phytochemical examination of the stem bark and leafy branches of *Ulmus pumila* L. gave rise to the separation of 13 compounds, recognized as Friedelin, 3*β*-acetoxyurs-11-en-13*β*, 28-olide, 3*β*-O-acetyl ursolic acid, 3*β*-O-acetyl oleanolic acid, *β*-sitosterol, stigmasterol, betulinic acid, methyl ursolate, methyl oleanolate, kaempferol-3-*O*-rutinoside, quercetin-3-*O*-*β*-D-glucopyranoside, quercetin-3-*O*-*β*-D-galactopyranoside, and caffeic acid. Their structures were elucidated using chemical and spectroscopic methods (ultraviolet, Infrared, EI-MS, ^{1}H-NMR, and ^{13}C-NMR) and by comparison with literature data. The cytotoxic potential of the crude methanol extract of the stem bark, besides the isolated triterpenoids, was tested against five human carcinoma cell lines, namely human colorectal carcinoma (HCT-116), human breast adenocarcinoma (MCF-7), human hepatocellular carcinoma (HepG2), human osteosarcoma (HOS), and human pulmonary adenocarcinoma (A549) cell lines. Betulinic acid exhibited a cytotoxic potential against MCF-7, HCT-116, and A549 cell lines with half maximal inhibitory concentration (IC_{50}) values equal to 22.39 ± 0.09 µM, 22.29 ± 0.05 µM, and 42.33 ± 0.06 µM, respectively. Meanwhile, the remaining triterpenoids showed a cytotoxic potential against HCT-116 and MCF-7 cell lines, with IC_{50} values ranging from 48.91 ± 0.12 to 78.98 ± 0.07 µM. The demonstrated cytotoxic potential of betulinic acid suggests its use as a lead compound for anticancer therapy.

INTRODUCTION

The family Ulmaceae, commonly known as the elm family, comprises about 6 genera and 45 species (Encyclopaedia Britannica, 2019). Ulmaceae members are evergreen or deciduous trees and shrubs distributed throughout the north temperate zone. *Ulmus* species, about 35 in number, are primarily distributed in Asia, Europe, and North America (Richens, 1983; Watson and Dallwitz, 1992). Previous studies on genus *Ulmus* reported the presence of various types of phytoconstituents like terpenoids (Martín-Benito *et al*., 2011), steroids (Martín-Benito *et al*., 2011), phenolics (Zhou *et al*., 2017), and polysaccharides (Lee *et al*., 2018). From a bioactivity standpoint, *Ulmus* species were reported to exhibit antibiotic (You *et al*., 2013), antifungal (Burden and Kemp, 1984), antioxidant (Bora *et al*., 2017; Joo *et al*., 2014; Mina *et al*., 2016), anti-inflammatory (Joo *et al*., 2014; Mina *et al*., 2016), hepatoprotective (Boudaoud-Ouahmed *et al*., 2015), neuroprotective (So *et al*., 2019), antiangiogenic (Jung *et al*., 2007), cytotoxic (Wang *et al*., 2004; Wang *et al*., 2006), anticancer (Hamed *et al*., 2015), and antiviral (Hamed *et al*., 2015) effects.

Ulmus pumila L., renowned as Asiatic elm, Chinese elm, and dwarf elm, is a deciduous tree belonging to central Asia. In folk medicine, its leaf and stem bark extracts are employed as diuretic, demulcent, antipyretic, and laxative remedies (Duke and Ayensu, 1985). *Ulmus pumila* L. was reported to possess large amounts of phenols and flavonoids with potent antioxidant activities (Kim *et al*., 2010). In addition, previous studies on the constituents of the root bark of this species led to the characterization of two potentially cytotoxic sesquiterpenoids, namely, mansonones E and F (Wang *et al*., 2004), as well as various bioactive triterpenoids (Wang *et al*., 2006). Moreover, four triterpenoids, namely, oleanolic acid, friedelin, maslinic acid, and arjunolic acid, were also isolated from the methanol extract of *U. pumila* L. (Ghosh *et al*., 2012). Furthermore, a recent phytochemical study on the stem bark extract of *U. pumila* L. led to separation of Icariside E_4 which strongly prohibited nitric oxide generation in LPS-activated macrophages (Joo *et al*., 2014). As an extension to our

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interest in exploring bioactive compounds from natural sources, we described in this report the isolation and identification of 13 compounds from *U. pumila* L. stem bark and leafy branches. The cytotoxic activity of the stem bark methanol extract and some of the isolated triterpenoids against five human carcinoma cell lines was also reported.

MATERIALS AND METHODS

Plant material

The stem bark and leafy branches of *U. pumila* L. were gathered from Orman Botanical Garden, Giza, Egypt, in January and February 2018. A voucher specimen, encoded M131, was submitted to the National Research Centre herbarium, Giza, Egypt.

General experimental procedures

Vacuum liquid chromatography (VLC) was achieved with silica gel H 60 (E-Merck, Darmstadt, Germany) and polyamide 11 (E-Merck, Darmstadt, Germany). Preparative and analytical thin layer chromatography were carried out using silica gel (E-Merck, Darmstadt, Germany). Chromatograms were first visualized under ultraviolet (UV) light and then sprayed with 20% sulfuric acid in methanol or ferric chloride reagent. Column chromatography was performed using Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO). Infrared (IR) spectra were run on a JASCO FT/IR-6100 Fourier Transform IR Spectrometer (Oklahoma, USA). Mass spectra (MS) were acquired by means of a Thermo ISQ Single Quadrupole Mass Spectrometer (THERMO Scientific Corporation, USA). UV spectra were displayed on a Shimadzu Double Beam Spectrophotometer UV-1650 (Shimadzu, Japan). Nuclear magnetic resonance (NMR) spectra were obtained via a Bruker High Performance Digital FT-NMR-Spectrophotometer Avance III HD (^{1}H-NMR: 400 MHz, ^{13}C-NMR: 100 MHz, Bremen, Germany). Chemical shifts were expressed on the δ scale and tetramethylsilane was used as an internal standard.

Extraction and isolation of stem bark and leafy branch constituents

Air-dried powdered stem bark and leafy branches (1 and 1.2 kg, resp.) were separately extracted with methanol (5 l × 3) at room temperature. Upon vacuum evaporation, the stem bark extract yielded a reddish-brown residue (50 g) and the leafy branch extract yielded a dark green residue (110 g). A portion of each dried extract (45 g of stem bark extract and 100 g of leafy branch extract) was individually suspended in distilled water (500 ml) and then partitioned with dichloromethane (250 ml × 5), ethyl acetate (250 ml × 5), and water-saturated *n*-butanol (300 ml × 4), in succession. The solvent-free dichloromethane fraction (19 g) from the stem bark extract and ethyl acetate fraction from leafy branch extract (4.2 g) were subjected to VLC (silica gel 500 g, and polyamide 11 250 g, resp.).

Elution of the silica gel bed was started using *n*-hexane and continued with *n*-hexane with 5% increments of acetone up to 50%. Thirty fractions, 100 ml each, were collected and examined by TLC (solvent system, *n*-hexane-CH_2Cl_2-MeOH, 10:10:1 *v/v/v*; spraying reagent, 20% sulfuric acid, followed by heating at 110°C). Fractions eluted with 10% acetone, with compound **1** as the major component, were combined and rechromatographed on a Sephadex LH-20 column (eluent, CH_2Cl_2-MeOH 3:2 *v/v*) to yield pure compound **1** (80 mg). The 25% acetone fractions (similar TLC pattern, three major spots) were pooled and the solvent was evaporated. The residue was subjected to repeated PTLC (solvent system, *n*-hexane-CH_2Cl_2-MeOH 10:10:1 *v/v/v*, triple development), followed by repeated chromatography on Sephadex LH-20 columns (eluent, CH_2Cl_2-MeOH 3:2 *v/v*) to yield compound **2** (24 mg), compound **3** (26 mg) slightly contaminated with compound **4**, and a mixture of compounds **5** and **6** (12.5 mg). The 35% acetone fractions (similar TLC pattern, two major spots) were pooled, evaporated, and subjected to repeated PTLC (solvent system, *n*-hexane-CH_2Cl_2-MeOH, 10:10:1 *v/v/v*, triple development), followed by repeated purification on Sephadex LH-20 columns (eluent, CH_2Cl_2-MeOH 3:2 *v/v*) to yield compound **7** (4.5 mg) and a mixture of compounds **8** and **9** (11.5 mg).

Elution of the polyamide 11 bed was started with H_2O and then by 10% increments of MeOH up to 80%. Eighty fractions, 100 ml each, were collected and monitored by TLC (solvent system, EtOAc-MeOH-H_2O 30:5:4 *v/v/v*). Spots were detected in visible and UV (365 nm) lights, before and after exposure to ammonia vapor or spraying with ferric chloride. Fractions eluted with 10, 20, and 30% MeOH, with compound **10** as the major component, were mixed. After evaporating the solvent, the residue was subjected to column chromatography (Sephadex LH-20; eluent, H_2O-MeOH 1:1 *v/v*) to yield compound **10** (11.5 mg). Fractions eluted with 50% and 60% MeOH were combined based on TLC analysis. After solvent evaporation, the residue was chromatographed on a Sephadex LH-20 column (eluent, H_2O-MeOH 1:1 *v/v*) to yield a mixture of compounds **11** and **12** (41.1 mg) together with compound **13** (10.5 mg).

Identification of the isolated compounds

Compounds **1–13** shown in Figure 1 were identified based on the following spectral data:

Friedelin (compound 1): EI-MS (*m/z*, relative abundance); 426 ([M]$^+$, $C_{30}H_{50}O$,2%), 411 ([M-Me]$^+$, 1%), 341 (1%), 273 (18%), 205 (28%), 123 (52%), 55 (100%). **IR (KBr, cm^{-1})**; 2,930, 2,868 (ν_{C-H}), 1,710 ($\nu_{C=O}$), 1,453, 1,385 (ν_{C-H}). **^{1}H-NMR ($CDCl_3$, ppm)**; 0.73 (3H, *s*, Me-24), 0.88 (3H, *s*, Me-25), 0.89 (3H, *d*, *J* = 6.0 Hz, Me-23), 0.96 (3H, *s*, Me-29), 1.02 (6H, *s*, Me-26, Me-30), 1.06 (3H, *s*, Me-27), 1.19 (3H, *s*, Me-28), 1.69 (1H, *m*, H-1a), 1.97 (1H, *m*, H-1b), 2.26 (1H, *q*, *J* = 6.4 Hz, H-4), 2.32 (1H, *m*, H-2a), 2.40 (1H, *m*, H-2b). **^{13}C-NMR ($CDCl_3$, ppm)**; 22.3 (C-1), 41.5 (C-2), 213.2 (C-3), 58.2 (C-4), 42.1 (C-5), 41.3 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.5 (C-10), 35.6 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.4 (C-15), 36.0 (C-16), 30.0 (C-17), 42.8 (C-18), 35.3 (C-19), 28.2 (C-20), 32.8 (C-21), 39.3 (C-22), 6.8 (C-23), 14.7 (C-24), 18.0 (C-25), 20.3 (C-26), 18.7 (C-27), 32.1 (C-28), 35.0 (C-29), 31.8 (C-30).

3*β*-acetoxyurs- 11-en-13*β*, 28-olide (compound 2): EI-MS (*m/z*, relative abundance); 496 ([M]$^+$, $C_{32}H_{48}O_4$, 0.2%), 452 ([M-CO_2]$^+$, 0.1%), 436 ([M-CH_3COOH]$^+$, 0.5%), 249 (2%), 248 (3%), 203 (6%), 165 (47%), 135 (37%), 123 (45%), 109 (68%), 81 (60%), 69 (100%). **IR (KBr, cm^{-1})**; 2,925, 2,856 (ν_{C-H}), 1,756 (sh.) ($\nu_{C=O}$, γ-lactone), 1,732 ($\nu_{C=O}$, ester), 1,645 ($\nu_{C=C}$), 1,460, 1,378 (ν_{C-H}), 1,243 (ν_{C-O}, acetate), 1,023 (ν_{C-O}). **^{1}H-NMR ($CDCl_3$, ppm)**; 0.87 (6H, *s*, Me-23, Me-25), 0.93 (3H, *d*, *J* = 5.5 Hz, Me-29), 0.98 (3H, *s*, Me-27), 1.09 (3H, *d*, *J* = 4.0 Hz, Me-30), 1.18

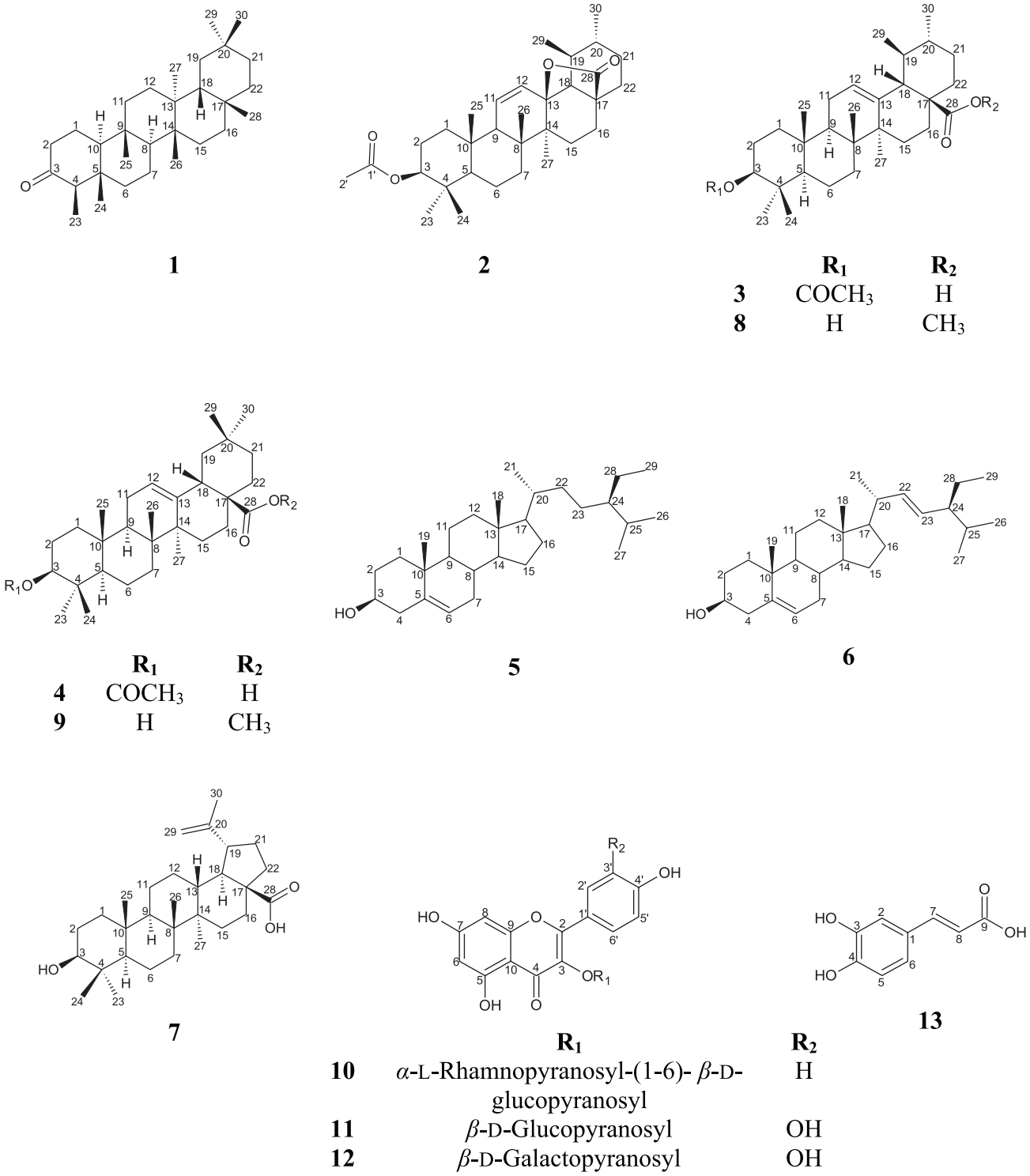

Figure 1. Phytoconstituents identified in *U. pumila* L. grown in Egypt.

(6H, *s*, Me-24, Me-26), 1.99 (3H, *s*, Acetate Me), 4.43 (1H, *dd*, *J* = 10.3, 5.8 Hz, H-3), 5.47 (1H, *dd*, *J* = 10.3, 2.7 Hz, H-11), 5.88 (1H, *d*, *J* = 10.3 Hz, H-12), **^{13}C-NMR ($CDCl_3$, ppm)**; 38.1 (C-1), 23.3 (C-2), 80.6 (C-3), 37.8 (C-4), 54.8 (C-5), 18.0 (C-6), 31.2 (C-7), 41.9 (C-8), 52.9 (C-9), 36.3 (C-10), 128.9 (C-11), 133.3 (C-12), 89.6 (C-13), 41.7 (C-14), 25.5 (C-15), 22.8 (C-16), 45.1 (C-17), 60.6 (C-18), 38.0 (C-19), 40.3 (C-20), 30.8 (C-21), 31.3 (C-22), 27.7 (C-23), 16.1 (C-24), 16.1 (C-25), 18.9 (C-26), 17.9 (C-27), 179.9 (C-28), 17.6 (C-29), 19.2 (C-30), 171.0 (Acetate C = O), 21.3 (Acetate Me).

3*β*-*O*-acetyl ursolic acid (compound 3): EI-MS (*m/z*, relative abundance); 498 ([M]$^+$, $C_{32}H_{50}O_4$,17%), 454 ([M-

CO2]+, 6%), 438 ([M-CH3COOH]+, 1%), 249 (69%), 248 (58%), 235 (13%), 203 (98%), 202 (19%), 190 (26%), 189 (65%), 133 (100%), 123 (34%), 120 (94%), 109 (24%). **IR (KBr, cm^{-1})**; 3,431 (ν_{O-H}), 2,925, 2,854 (ν_{C-H}), 1,728 ($\nu_{C=O}$), 1,630 ($\nu_{C=C}$), 1,440, 1,383 (ν_{C-H}), 1,253 (ν_{C-O}, acetate), 1,025 (ν_{C-O}). **^{1}H-NMR (CDCl$_3$, ppm)**; 0.78 (3H, *s*, Me-27), 0.87 (3H, *s*, Me-26), 0.89 (3H, *s*, Me-25), 0.97 (6H, *d*, *J* = 6.6 Hz, Me-29, Me-30), 1.09 (3H, *s*, Me-24), 1.27 (3H, *s*, Me-23), 2.07 (3H, *s*, Acetate Me), 2.20 (1H, *d*, *J* = 11.2 Hz, H-18), 4.52 (1H, *m*, H-3), 5.25 (1H, *br s*, H-12). **^{13}C-NMR (CDCl$_3$, ppm)**; 38.3 (C-1), 23.6 (C-2), 81.0 (C-3), 37.7 (C-4), 55.3 (C-5), 18.2 (C-6), 32.8 (C-7), 39.5 (C-8), 47.5 (C-9), 36.9 (C-10), 23.3 (C-11), 125.7 (C-12), 138.0 (C-13), 41.9 (C-14), 28.0 (C-15), 24.0 (C-16), 48.0 (C-17), 52.5 (C-18), 38.8 (C-19), 39.0 (C-20), 30.6 (C-21), 36.7 (C-22), 28.1 (C-23), 17.1 (C-24), 15.5 (C-25), 16.7 (C-26), 23.6 (C-27), 184.0 (C-28), 17.0 (C-29), 21.2 (C-30), 171.1 (Acetate C = O), 21.3 (Acetate Me).

3*β*-*O*-acetyl oleanolic acid (compound 4): EI-MS (*m/z*, relative abundance); 498 ([M]$^+$, $C_{32}H_{50}O_4$,17%), 454 ([M-CO2]+, 6%), 438 ([M-CH3COOH]+, 1%), 249 (69%), 248 (58%), 235 (13%), 203 (98%), 202 (19%), 190 (26%), 189 (65%), 133 (100%), 123 (34%), 120 (94%), 109 (24%). **IR (KBr, cm^{-1})**; 3,431 (ν_{O-H}), 2,925, 2,854 (ν_{C-H}), 1,728 ($\nu_{C=O}$), 1,630 ($\nu_{C=C}$), 1,440, 1,383 (ν_{C-H}), 1,253 (ν_{C-O}, acetate), 1,025 (ν_{C-O}). **^{1}H-NMR (CDCl$_3$, ppm)**; 0.76 (3H, *s*, Me-26), 0.84 (6H, *s*, Me-23, Me-24), 0.92 (3H, *s*, Me-30), 0.95 (6H, *s*, Me-25, Me 29), 1.14 (3H, *s*, Me-27), 2.07 (3H, *s*, Acetate Me), 4.52 (1H, *m*, H-3), 5.29 (1H, *br s*, H-12). **^{13}C-NMR (CDCl$_3$, ppm)**; 38.1 (C-1), 23.6 (C-2), 81.0 (C-3), 37.7 (C-4), 55.3 (C-5), 18.2 (C-6), 32.6 (C-7), 39.3 (C-8), 47.5 (C-9), 36.9 (C-10), 22.8 (C-11), 122.5 (C-12), 143.6 (C-13), 41.6 (C-14), 27.7 (C-15), 23.4 (C-16), 46.6 (C-17), 40.9 (C-18), 45.8 (C-19), 30.7 (C-20), 33.8 (C-21), 32.5 (C-22), 28.1 (C-23), 16.7 (C-24), 15.4 (C-25), 17.2 (C-26), 25.9 (C-27), 184.3 (C-28), 33.1 (C-29), 23.6 (C-30), 171.1 (Acetate C = O), 21.3 (Acetate Me).

***β*-Sitosterol (compound 5): EI-MS (*m/z*, relative abundance)**; 414 (**[M]+**, $C_{29}H_{50}O$, 85%), 399 (**[M-Me]+**, 14%); 396 (**[M-H2O]+**, 19%), 381 (30%), 273 (4%), 255 (28%), 213 (80%), 161 (31%), 133 (100%), 105 (83%). **^{1}H-NMR (CDCl$_3$, ppm)**; 0.70 (3H, *s*, Me-19), 0.83–0.89 (9H, *m*, Me-26, Me-27, Me-29), 0.94 (3H, *d*, *J* = 6.6 Hz, Me-21), 1.03 (3H, *s*, Me-18), 3.67 (1H, *m*, H-3), 5.37 (1H, *d*, *J* = 4.6 Hz, H-6).

Stigmasterol (compound 6): EI-MS (*m/z*, relative abundance); 412 ([M]$^+$, $C_{29}H_{48}O$,8%), 397 (**[M-Me]**$^+$, 28%), 394 (**[M-H_2O]**$^+$, 1%), 379 (0.01%), 351 (0.1%), 273 (4%), 271 (9%), 257 (2%), 255 (28%), 229 (36%), 213 (80%), 133 (100%), 107 (79%), 105 (83%). **^{1}H-NMR (CDCl$_3$, ppm)**; 0.70 (3H, *s*, Me-19), 0.83–0.89 (9H, *m*, Me-26, Me-27, Me-29), 0.94 (3H, *d*, *J* = 6.6 Hz, Me-21), 1.31 (3H, *s*, Me-18), 3.55 (1H, *m*, H-3), 5.04 (1H, *dd*, *J* = 16.0, 8.0 Hz, H-22), 5.18 (1H, *dd*, *J* = 16.0, 8.0 Hz, H-23), 5.37 (1H, *d*, *J* = 4.6 Hz, H-6).

Betulinic acid (compound 7): EI-MS (*m/z*, relative abundance); 456 ([M]$^+$, $C_{30}H_{48}O_3$,5%), 441 ([M-Me]$^+$, 6%), 411 ([M-COOH]$^+$, 0.04%), 248 (26%), 220 (31%), 207 (27%), 203 (43%), 189 (100%), 187 (31%), 175 (18%), 173 (27%), 135 (82%), 119 (94%). **IR (KBr, cm^{-1})**; 3,439 (ν_{O-H}), 2,926 and 2,860 (ν_{C-H}), 1,678 (sh., $\nu_{C=O}$), 1,641 ($\nu_{C=C}$), 1,456, 1,381 (ν_{C-H}), 1,267, 1,027 (ν_{C-O}). **^{1}H-NMR (CDCl$_3$, ppm)**; 0.68 (3H, *s*, Me-24), 0.75 (3H, *s*, Me-25), 0.87 (3H, *s*, Me-23), 0.90 (3H, *s*, Me-27), 0.91 (3H, *s*, Me-26), 1.62 (3H, *s*, Me-30), 1.90 (1H, *m*, H-18), 2.93 (1H, *td*, *J* = 10.6, 4.7 Hz, H-19), 3.12 (1H, *dd*, *J* = 11.1, 4.8 Hz, H-3), 4.54 (1H, *br s*, H-29a), 4.67 (1H, *br s*, H-29b).

Methyl ursolate (compound 8): EI-MS (*m/z*, relative abundance); 470 ([M]$^+$, $C_{31}H_{50}O_3$,25%), 262 (1%), 249 (51%), 208 (0.1%), 203 (18%), 191 (22%), 189 (5%), 175 (27%), 133 (27%), 123 (1%), 120 (100%), 109 (29%). **IR (KBr, cm^{-1});** 3,427 (ν_{O-H}), 2,925, 2,856 (ν_{C-H}), 1,730 (sh., $\nu_{C=O}$), 1,631 ($\nu_{C=C}$), 1,458, 1,381 (ν_{C-H}), 1,277, 1,029 (ν_{C-O}). **^{1}H-NMR (CDCl$_3$, ppm)**; 0.76 (3H, *s*, Me-27), 0.86 (3H, *s*, Me-26), 0.88 (3H, *s,* Me-25), 0.93 (6H, *d*, *J* = 6.8 Hz, Me-29, Me-30), 1.10 (3H, *s*, Me-24), 1.26 (3H, *s*, Me-23), 2.12 (1H, *d*, *J* = 11.3 Hz, H-18), 3.15 (1H, *dd*, *J* = 10.3, 4.3 Hz, H-3), 3.60 (3H, *s*, MeOOC-28) and 5.18 (1H, *br s*, H-12). **^{13}C-NMR (CDCl$_3$, ppm)**; 38.8 (C-1), 27.2 (C-2), 79.1 (C-3), 38.8 (C-4), 55.2 (C-5), 18.3 (C-6), 32.9 (C-7), 39.5 (C-8), 47.5 (C-9), 37.1 (C-10), 17.0 (C-11), 125.7 (C-12), 138.0 (C-13), 42.0 (C-14), 28.1 (C-15), 24.7 (C-16), 48.0 (C-17), 52.6 (C-18), 39.3 (C-19), 38.8 (C-20), 30.6 (C-21), 36.7 (C-22), 28.1 (C-23), 15.5 (C-24), 15.6 (C-25), 17.0 (C-26), 23.4 (C-27), 178.3 (C-28), 23.6 (C-29), 21.2 (C-30), 51.5 (MeOOC-28).

Methyl oleanolate (compound 9): EI-MS (*m/z*, relative abundance); 470 ([M]$^+$, $C_{31}H_{50}O_3$, 25%), 262 (1%), 249 (51%), 208 (0.1%), 203 (18%), 191 (22%), 189 (5%), 175 (27%), 133 (27%), 123 (1%), 120 (100%), 109 (29%). **IR (KBr, cm^{-1})**; 3,427 (ν_{O-H}), 2,925, 2,856 (ν_{C-H}), 1,730 (sh., $\nu_{C=}$), 1,631 ($\nu_{C=C}$), 1,458, 1,381 (ν_{C-H}), 1,277, 1,029 (ν_{C-O}). **^{1}H-NMR (CDCl$_3$, ppm)**; 0.71 (3H, *s*, Me-26), 0.80 (3H, *s*, Me-24), 0.87 (3H, *s,* Me-23), 0.88 (3H, *s*, Me-30), 0.92 (3H, *s*, Me-29), 1.01 (3H, *s*, Me-25), 1.07 (3H, *s*, Me-27), 2.76 (1H, *m*, H-18), 3.15 (1H, *dd*, *J* = 10.3, 4.3 Hz, H-3), 3.60 (3H, *s*, MeOOC-28), 5.21 (1H, *br s*, H-12). **^{13}C-NMR (CDCl$_3$, ppm)**; 38.4 (C-1), 27.2 (C-2), 79.1 (C-3), 38.8 (C-4), 55.2 (C-5), 18.3 (C-6), 32.8 (C-7), 39.3 (C-8), 47.5 (C-9), 37.1 (C-10), 23.1 (C-11), 122.8 (C-12), 143.6 (C-13), 41.7 (C-14), 28.0 (C-15), 23.4 (C-16), 46.6 (C-17), 41.1 (C-18), 45.8 (C-19), 30.6 (C-20), 33.8 (C-21), 32.1 (C-22), 28.1 (C-23), 15.5 (C-24), 15.4 (C-25), 17.0 (C-26), 25.9 (C-27), 178.6 (C-28), 33.0 (C-29), 23.6 (C-30), 51.5 (MeOOC-28).

Kaempferol-3-*O*-rutinoside (nicotiflorin, compound 10): UV spectral data (nm); 267, 302sh., 353 (CH_3OH), 276, 329, 404 (inc.) (CH_3ONa), 276, 306sh., 350, 397 ($AlCl_3$), 277, 346, 392 ($AlCl_3$/HCl), 274, 305, 370 (CH_3COONa), 267, 354 (CH_3COONa /H_3BO_3). **^{1}H-NMR [(CD_3)$_2$CO + D_2O, ppm];** 6.27 (1H, *d*, *J* = 1.6 Hz, H-6), 6.52 (1H, *d*, *J* = 1.6 Hz, H-8), 6.97 (2H, *d*, *J* = 8.8 Hz, H-3′,5′), 8.12 (2H, *d*, *J* = 8.8 Hz, H-2′,6′), 5.14 (1H, *d*, *J* = 7.2 Hz, H-1″), 4.56 (1H, *br s*, H-1‴), 1.09 (3H, *d*, *J* = 6.1 Hz, Me-6‴), 3.37–3.63 (Sugar protons).

Quercetin-3-*O*-*β*-D-glucopyranoside (isoquercetin, compound 11): UV spectral data (nm); 258, 270sh., 299sh., 362 (CH_3OH), 273, 330, 409 (inc.) (CH_3ONa), 273, 304sh., 370sh., 416 ($AlCl_3$), 270, 299sh., 367sh., 363 ($AlCl_3$/HCl), 272, 387 (CH_3COONa), 262, 378 (CH_3COONa /H_3BO_3). **^{1}H-NMR [(CD_3)$_2$CO + D_2O, ppm]** 6.23 (1H, *br s*, H-6), 6.47 (1H, *br s*, H-8), 6.93 (1H, *br s*, H-5′), 7.56 (1H, *d*, *J* = 7.9 Hz, H-6′), 7.80 (1H, *br s*, H-2′), 5.23 (1H, *d*, *J* = 6.4 Hz, H-1″), 3.25–3.93 (Sugar protons). **^{13}C-NMR [(CD_3)$_2$CO + D_2O, ppm]**; 157.3 (C-2), 134.4 (C-3), 178.1 (C-4), 161.2 (C-5), 98.9 (C-6), 164.5 (C-7), 94.0 (C-8), 157.7 (C-9), 104.3 (C-10), 121.6 (C-1′), 116.8 (C-2′), 144.4

(C-3′), 148.4 (C-4′), 115.3 (C-5′), 121.7 (C-6′), 102.8 (C-1″), 74.1 (C-2″), 76.3 (C-3″), 69.4 (C-4″), 76.6 (C-5″), 60.9 (C-6″).

Quercetin-3-*O*-*β*-D-galactopyranoside (hyperoside, compound 12): UV spectral data (nm); 258, 270sh., 299sh., 362 (CH_3OH), 273, 330, 409 (inc.) (CH_3ONa), 273, 304sh., 370sh., 416 ($AlCl_3$), 270, 299sh., 367sh., 363 ($AlCl_3$/HCl), 272, 387(CH_3COONa),262, 378 (CH_3COONa /H_3BO_3). **^{1}H-NMR [$(CD_3)_2CO$ + D_2O, ppm]**; 6.23 (1H, *br s*, H-6), 6.47 (1H, *br s*, H-8), 6.93 (1H, *br s*, H-5′), 7.56 (1H, *d*, *J* = 7.9 Hz, H-6′), 7.92 (1H, *br s*, H-2′), 5.13 (1H, *d*, *J* = 7.7 Hz, H-1″), 3.25−3.93 (Sugar protons). **^{13}C-NMR [$(CD_3)_2CO$ + D_2O, ppm]**; 157.7 (C-2), 134.2 (C-3), 178.0 (C-4), 161.2 (C-5), 98.9 (C-6), 164.5 (C-7), 94.0 (C-8), 156.9 (C-9), 104.3 (C-10), 121.4 (C-1′), 116.6 (C-2′), 144.4 (C-3′), 148.5 (C-4′), 115.3 (C-5′), 122.0 (C-6′), 103.7 (C-1″), 71.7 (C-2″), 73.4 (C-3″), 68.1 (C-4″), 75.4 (C-5″), 60.1 (C-6″).

3, 4-Dihydroxycinnamic acid (caffeic acid, compound 13): UV spectral data (nm); 204sh., 220, 242, 298, 326 (CH_3OH). **^{1}H-NMR [$(CD_3)_2CO$, ppm]**; 6.22 (1H, *d*, *J* = 15.9 Hz, H-8), 6.83 (1H, *d*, *J* = 8.2 Hz, H-5), 6.96 (1H, *dd*, *J* = 8.1, 1.8 Hz, H-6), 7.10 (1H, *d*, *J* = 1.8 Hz, H-2), 7.47 (1H, *d*, *J* = 15.9 Hz, H-7).

Acid hydrolysis of glycosides

Aliquots (3 mg, each) of the isolated glycosides were separately subjected to acid hydrolysis. The sample was dissolved in 3 ml of 2N hydrochloric acid-methanol mixture (1:1 *v/v*) and heated under reflux on a water bath for 2 hours. The reaction mixture was further evaporated under vacuum to dryness, and the residue was suspended in distilled water (10 ml) and then repeatedly extracted with ethyl acetate. The ethyl acetate layer was subjected to TLC (solvent system, $CHCl_3$-MeOH 5:1 *v/v*) alongside reference aglycones. Meanwhile, the aqueous layer was diluted with methanol and evaporated to dryness and the residue obtained was investigated by PC and TLC- (solvent systems: *n*-butanol-acetic acid-water 4:1:5 *v/v/v*, upper layer; and isopropanol:water 7:1 *v/v*, resp.) for detection of sugar moieties (Mabry *et al*., 1970). Hydrolysis of compound **10** afforded D-glucose and L-rhamnose, while the mixture of compounds **11** and **12** yielded D-glucose and D-galactose.

Cytotoxic evaluation of the stem bark extract and isolated triterpenoids

Cell lines, culture media, and reference drug

The cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and included human breast adenocarcinoma (MCF-7), human colorectal carcinoma (HCT-116), human hepatocellular carcinoma (HepG2), human osteosarcoma (HOS), and human pulmonary adenocarcinoma (A549) cell lines, alongside telomerase-immortalized normal human retinal epithelial cell line- (RPE-1).

Cell culture was carried out under sterile conditions using a laminar airflow cabinet biosafety class II level. The culture was maintained in McCoy's 5a medium in case of HCT-116 cell line, Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 in case of A549 and RPE-1 cell lines, and Eagle's Minimum Essential Medium in case of MCF7, HepG2, and HOS cell lines. The culture media were supplied with 1% antibiotic-antimycotic mixture (10,000 U/ml potassium penicillin, 10,000 µg/ml streptomycin sulfate, and 25 µg/ml amphotericin B), 1% L-glutamine, and 10% heat-inactivated fetal bovine serum. Cisplatin was used as a positive control and 0.5% DMSO solution as a negative control (Thabrew *et al*., 1997).

Cell viability assay

The cells were seeded at concentrations of 10,000 cells/well in case of MCF-7, HepG2, A549, and HOS cell lines and 20,000 cells/well in case of HCT-116 and RPE-1 cell lines, using 96-well microtiter plastic plates at 37°C for 24 hours under 5% CO_2 in a carbon dioxide incubator. Stock solutions of the test isolates were prepared at concentrations of 20, 10, 5, and 2.5 mg/ml for the stem bark methanol extract and 20, 10, 5, and 2.5 mM for each isolate. Culture media were aspirated from the cell culture plates, and four different concentrations of the test isolates were prepared, in triplicates. This was carried out by adding an aliquot (1 µl) of each stock solution of the test isolates to fresh medium with cells (199 µl) in each well to reach final concentrations of 100, 50, 25, and 12.5 µg/ml for the crude extract and 100, 50, 25, and 12.5 µM for each isolate. After incubation for 72 hours, the media were aspirated and 40 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt (2.5 µg/ml) was added to each well followed by 4 h. incubation at 37ºC under 5% CO_2. In order to stop the reaction and dissolve the formed crystals, 200 µl of 10% solution of sodium dodecyl sulfate in deionized water was added to each well, followed by incubation overnight, at 37°C. The absorbance was measured using a microplate multiwell reader at 595 nm. Cell viability was determined using a modified procedure of MTT assay, based on mitochondrial-dependent reduction of the yellow MTT to purple formazan (Mosmann, 1983). The cytotoxicity percentage was calculated as follows:

$$\% \text{ Cytotoxicity} = [1 - (Avg_X / Avg_{NC})] \times 100,$$

where Avg indicates average; X indicates absorbance of sample, and NC indicates absorbance of the negative control.

The half maximal inhibitory concentration (IC_{50}) values of the tested samples were calculated using GraphPad Prism software (version 5.0.), and the selectivity indices (SI) of the cytotoxic samples were deduced from the following equation (Pritchett *et al*., 2014):

SI = IC_{50} of sample against normal cell line/IC_{50} of sample against cancer cell line.

The IC_{50} values and SI of the methanol extract of the stem bark, triterpenoidal isolates, and positive control (cisplatin) against the tested cell lines are recorded in Tables 1 and 2.

RESULTS AND DISCUSSION

In an attempt to explore natural compounds with cytotoxic activity, the phytochemical composition of the Egyptian cultivar of *U. pumila* L. was investigated and its cytotoxic potential was assessed. Six compounds, namely, 3*β*-acetoxyurs-11-en-13*β*,28-olide, 3*β*-*O*-acetyl ursolic acid, 3*β*-*O*-acetyl oleanolic acid, methyl ursolate, methyl oleanolate, and hyperoside, represented the first reported occurrence in genus *Ulmus*, whereas the two compounds betulinic acid and nicotiflorin were described from *U. pumila* L. for the first time. Friedelin (Martín-Benito *et al*., 2011; Wang

Table 1. IC_{50} values of the tested samples.

Tested samples	IC_{50}					
	MCF-7	HCT-116	HepG2	HOS	A549	RPE-1*
Methanol extract	29.54 ± 0.05 µg/ml	> 100 µg/ml	> 100 µg/ml	> 100 µg/ml	> 100 µg/ml	> 100 µg/ml
Friedelin	> 100 µM	> 100 µM	> 100 µM	> 100 µM	> 100 µM	> 100 µM
3*β*-Acetoxyurs-11-en-13*β*,28-olide	> 100 µM	78.98 ± 0.07 µM	> 100 µM	> 100 µM	> 100 µM	> 100 µM
3*β*-*O*-Acetyl ursolic acid and/or 3*β*-*O*-acetyl oleanolic acid	> 100 µM	48.91 ± 0.12 µM	> 100 µM	> 100 µM	> 100 µM	> 100 µM
Betulinic acid	22.39 ± 0.09 µM	22.29 ± 0.05 µM	> 100 µM	> 100 µM	42.33 ± 0.06 µM	31.14 ± 0.06 µM
Methyl ursolate and/or methyl oleanolate	50.71 ± 0.18 µM	52.96 ± 0.07 µM	> 100 µM	> 100 µM	> 100 µM	69.43 ± 0.2 µM

*Normal cell line.

IC_{50} = half maximal inhibitory concentration; MCF-7 = human breast adenocarcinoma; HCT-116 = human colorectal carcinoma; HepG2 = human hepatocellular carcinoma; HOS = human osteosarcoma; A549 = human pulmonary adenocarcinoma; and RPE-1 = telomerase-immortalized normal human retinal epithelial cell line.

Table 2. SI of the cytotoxic samples.

Tested samples	SI		
	MCF-7	HCT-116	A549
Methanol extract	> 3.4	Inactive	Inactive
3*β*-Acetoxyurs-11-en-13*β*,28-olide	Inactive	> 1.3	Inactive
3*β*-*O*-Acetyl ursolic acid and/or 3*β*-*O*-acetyl oleanolic acid	Inactive	> 2.0	Inactive
Betulinic acid	1.4	1.4	0.7
Methyl ursolate and/or methyl oleanolate	1.4	1.3	Inactive

MCF-7 = human breast adenocarcinoma; HCT-116 = human colorectal carcinoma; and A549 = human pulmonary adenocarcinoma.

et al., 2006; Zheng *et al*., 2010), *β*-sitosterol, stigmasterol (Martín-Benito *et al*., 2011; Zheng *et al*., 2010), isoquercetin (Santamour, 1972; Sherman and Giannasi, 1988), and caffeic acid (Zhou *et al*., 2017) were previously reported from different *Ulmus* species.

Structure elucidation of the isolates was performed based on spectral analyses (UV, IR, EI-MS, ^{1}H-, and ^{13}C-NMR) and by comparing the data with literature values. Compounds obtained as pure isolates included friedelin (compound **1**) (Mann *et al*., 2012), 3*β*-acetoxyurs-11-en-13*β*,28-olide (compound **2**) (Raza *et al*., 2015), betulinic acid (compound **7**) (Lee *et al*., 2005), kaempferol-3-*O*-rutinoside (nicotiflorin) (compound **10**) (Erosa-Rejón *et al*., 2010), and 3,4-dihydroxycinnamic acid (caffeic acid) (compound **13**) (Zhou *et al*., 2017). In accordance with previous reports (Basir *et al*., 2014), 3*β*-*O*-acetyl ursolic acid (compound **3**) was slightly contaminated with 3*β*-*O*-acetyl oleanolic acid (compound **4**). Furthermore, the isolation of *β*-sitosterol and stigmasterol (compounds **5** and **6**), methyl ursolate, and methyl oleanolate (compounds **8** and **9**) as well as quercetin-3-*O*-*β*-D-glucopyranoside (isoquercetin) and quercetin-3-*O*-*β*-D-galactopyranoside (hyperoside) (compounds **11** and **12**) as mixtures was in agreement with earlier studies (Furuya *et al*., 1987; Luhata and Munkombwe, 2015; Pereira *et al*., 2011).

The cytotoxic efficiency of the stem bark methanol extract (Table 1) might be ascribed to its triterpenoidal components as many of these constituents play an essential role in the upregulation and downregulation of several important genes that influence the apoptotic effects (Prabhu *et al*., 2011). The isolated betulinic acid was moderately active against **MCF-7**, **HCT-116**, and **A549** cell lines (respective IC_{50} values: 22.39 ± 0.09, 22.29 ± 0.05, and 42.33 ± 0.06 µM); nevertheless, it was insufficiently selective to MCF-7 and HCT-116 cell lines (SI = 1.4) and lacked selectivity to A549 cell line (SI = 0.7). The cytotoxic potential of betulinic acid was previously explained by its ability to induce apoptosis by directly affecting the mitochondria leading to cleavage of caspase-9 and caspase-3 and activation of nuclear factor-kappa-B (NF-kappa-B), which is a key regulator of stress-induced transcriptional activation (Tripathi *et al*., 2009). Furthermore, betulinic acid was found to inhibit angiogenesis and metastatic activity through inhibition of aminopeptidase N enzyme (Melzig and Bormann, 1998). Studying the structure-activity relationship of betulinic acid revealed that the skeleton composed of rings A, B, and C as well as the carboxylic acid function at C-28 was essential for eliciting its cytotoxic activity (Mukherjee *et al*., 2006).

CONCLUSION

The variability in triterpenoidal composition between locally acclimatized *U. pumila* L. samples and those obtained from plants growing abroad could be attributed to environmental conditions. Moreover, the established cytotoxic efficiency of betulinic acid suggests its use as a lead compound for synthesizing potential cytotoxic agents.

AUTHORS' CONTRIBUTIONS

All authors have contributed to gathering literature data, carrying out the chemical and spectral analyses, interpreting the

results, and drafting of this manuscript. All authors have read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare that there are no competing interests.

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