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Original article

Maslinic acid derivatives induce significant apoptosis in b16f10 murine melanoma cells

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ABSTRACT

Maslinic acid (2α,3β-dihydroxyolean-12-en-28-oic acid), a natural dihydroxylated pentacyclic triterpene acid isolated from olive-pressing residues, has been investigated together with some of its derivatives regarding the induction of apoptosis in B16F10 melanoma cells. Some of the compounds tested are described in this work, but others come from previous studies. Ten of these derivatives induce over 80% of apoptosis, clearly promoting cell death in B16F10 melanoma. By contrast, the induction cell death through necrosis was very slightly significant with these compounds. These results indicate that maslinic acid derivatives are promising chemopreventive and chemotherapeutic agents.

1. Introduction

Natural products have a noteworthy role in drug discovery, as almost half of all new drugs are natural compounds or directly derived therefrom [1–4]. Triterpenoids are compounds derived from squalene and widely present in plants used in traditional medicine [5–9]. Tetra- and pentacyclic triterpenoids display anti-inflammatory, anti-microbial, anti-HIV, anti-oxidant, hepato-protective, and analgesic effects [10–13]. However, the disadvantage of using many of these triterpenoids is the toxicity due to their haemolytic and cytostatic properties. In addition, several natural triterpene compounds and some derivatives induce apoptosis in a wide variety of cancer cells, such as breast carcinoma, melanoma, hepatoma, prostate carcinoma and leukaemia [14–17]. Apoptosis is a programmed process of cell death, and inappropriately regulated apoptosis is involved in diverse disease states, such as neuro-degenerative disease and cancer. Agents that suppress the proliferation of malignant cells, and even cause apoptosis, have the potential to both prevent and treat cancer. The identification of new cytotoxic agents that enhance or restore the capability of malignant cancer cells to undergo apoptosis may be crucial for more effective anticancer therapies.

Oleanolic acid (OA, **1**, 3β-hydroxyolean-12-en-28-oic acid) and maslinic acid (MA, **2**, 2α,3β-dihydroxyolean-12-en-28-oic acid) (Fig. 1) are two pentacyclic triterpenes present in high concentrations in olive-pomace oil, being the main components of the protective wax-like coating of the olive skin [18,19]. A method to obtain large amounts of both compounds from the olive-pressing residues has been reported by our group [20]. Maslinic acid (MA, **2**) has been found to reduce cell proliferation and promote cell apoptosis in various types of human cancer [21–28]. Moreover, in the last decade, our research group has published several papers on the reactivity and some biological activities of OA and MA [29–35].

In the present paper, we have semi-synthesized a series of MA derivatives (**3**–**21**), which have been tested for their apoptosis-inducing abilities on B16F10 melanoma cells. This apoptotic effect was also investigated for a set of triterpene compounds previously developed in our laboratory (**22**–**40**). Some of these MA derivatives exhibit strongly apoptotic effects without any cytotoxic consequence.

2. Results and discussion

2.1. Chemistry

Starting with natural maslinic acid (MA, **2**), we prepared several derivatives to be tested as potential apoptotic agents. Thus, compound **2** was transformed into its corresponding sodium salt

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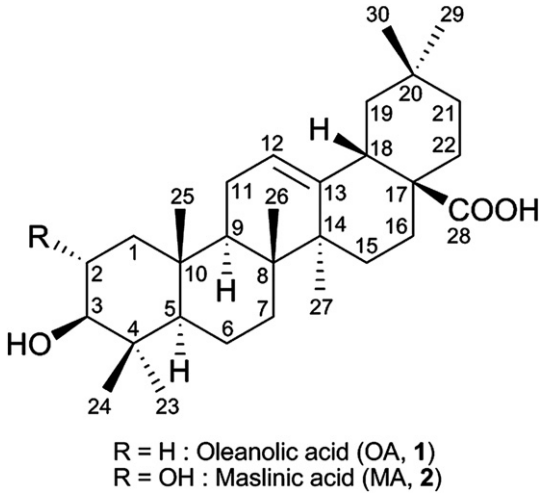


Fig. 1. Compounds **1** and **2**.

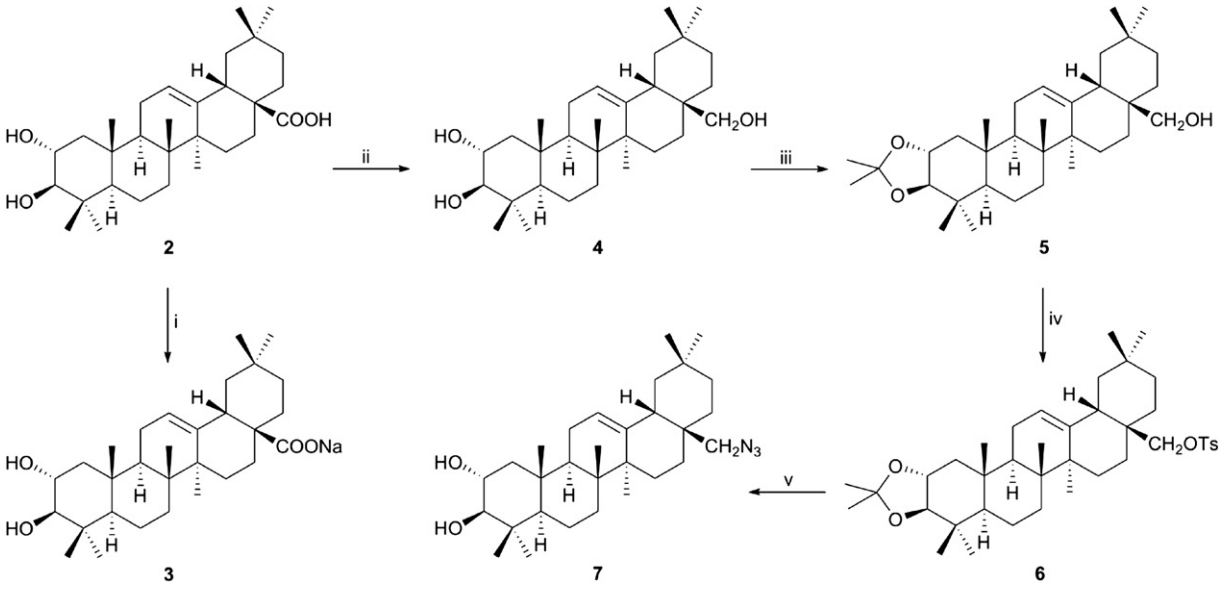
(**3**) by several procedures (see Experimental). This derivative **3** presented NMR data very similar to that of MA (**2**), differing only in its water solubility. Moreover, MA (**2**) was treated with $LiAlH_4$ in THF to form 2α-hydroxyerythrodiol (**4**) [36]. The subsequent reaction of this triol (**4**) with 2,2-dimethoxypropane produced the ketal derivative **5**, in which both hydroxyl groups of the A-ring were blocked. The tosyl derivative **6** was formed by treatment with tosyl chloride in pyridine, and converted into the desired compound **7**, which has a $-N_3$ group on C-28, through an azidation reaction (Scheme 1).

Structures of these compounds **5**–**7** can be easily deduced from their ^1H and ^{13}C NMR data. The NMR signals for compound **5** are compatible with the presence of an acetonide group between C-2 and C-3 (δ 1.38 and 1.40 in its ^1H NMR spectrum and δ 27.1, 27.4 and 108.4 in its ^{13}C NMR spectrum). Compound **6** had the same NMR signals as compound **5** had, except for the corresponding signals of the tosyl group on C-28 (δ 2.42, 7.30 and 7.74 in its ^1H NMR spectrum). Finally, the ^1H and ^{13}C NMR spectra of **7** revealed the absence of acetonide and tosyl groups as well as the presence of a $-N_3$ group on C-28 (δ 69.9).

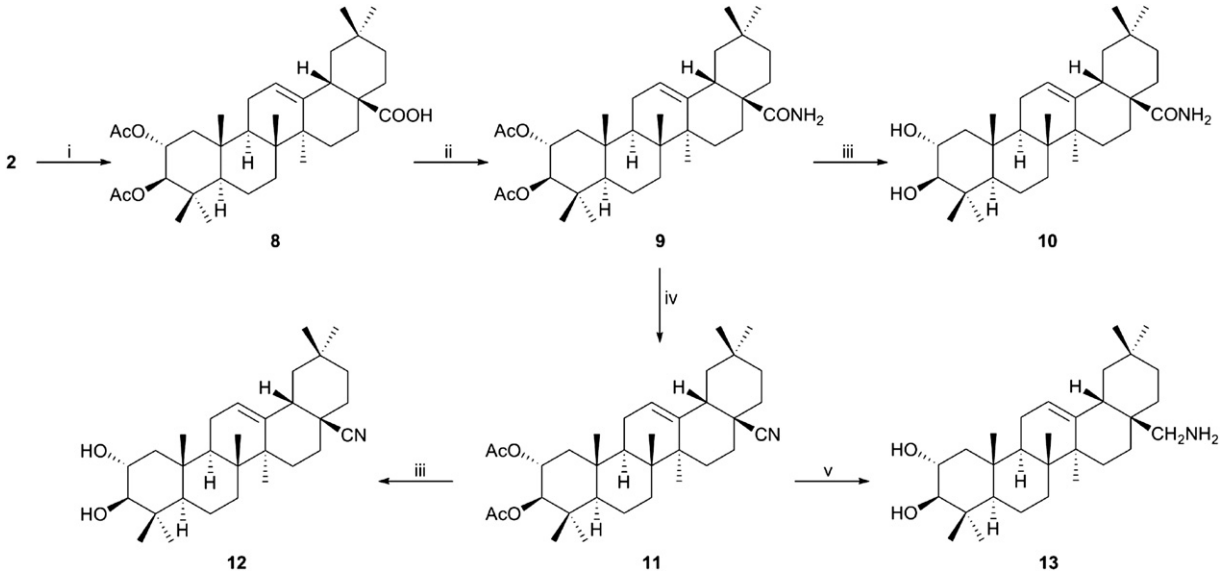
Several triterpenoids containing a nitrogen atom on C-28 have shown significant biological activities [37,38]. On this basis, we prepared a new series of MA derivatives with a nitrogen atom on C-28, starting from this natural product (Scheme 2). Thus, MA (**2**) was acetylated to obtain the diacetyl derivative **8** [39], which was transformed into the corresponding amide derivative **9** by treatment, firstly with thionyl chloride/DCM and then with $MeOH/NH_3$. The diacetyl amide **9** was deacetylated to form compound **10**. The ^{13}C NMR spectra of these amide derivatives (**9** and **10**) differed mainly from that of **8** in the signal of C-28, because it is more shielded in a carboxylic acid group (δ 184.5 for **8**) than in an amide group (δ 182.2 for **9** and 181.5 for **10**). The amide derivative **9** was also converted into the nitrile derivative **11** by a treatment with thionyl chloride in DCM, which was then deacetylated to form compound **12**. In the ^{13}C NMR spectra of both nitrile derivatives (**11** and **12**), the C-28 signals are the most relevant (δ 125.7 for **11** and 125.8 for **12**). Finally, the amine derivative **13** was prepared by the treatment of **11** with $LiAlH_4$ in THF. The NMR signals, for **13**, of the methylene group at C-28 (δ 3.23 and 3.57) and the signal of this carbon atom (δ 69.9) confirmed the presence of an amine group.

Scheme 3 shows the third set of MA (**2**) derivatives that were also tested against B16F10 melanoma cells. Thus, we treated MA (**2**) with PCC/DCM to obtain a partially oxidized product (**14**, 40%), and a previously described conjugated dicarbonyl product (**15**, 40%) [39], which appeared in its ketoenolic form. Triterpenoids with the same conjugated dicarbonylic system on the A-ring have proved to be cytotoxic against several cancer-cell lines [40]. The NMR data of **14** (a singlet signal of 3-H at δ 3.92 and a signal of a carbonyl group on C-2 at δ 211.2) confirmed the presence of a carbonyl group on C-2, as a result of the oxidation of the hydroxyl group on this carbon of MA (**2**). In addition, to test the influence that the configuration of the hydroxyl group at C-2 exerted on the apoptotic activity, compound **14** was reduced with $NaBH_4$ to form the 2-*epi*-maslinic acid (augustic acid, **16**). The narrow ^1H NMR signal of the geminal proton of the hydroxyl group at C-2 (δ 4.08, ddd, *J* 3.1, 3.1 and 4.4), for **16**, confirmed the configuration in this carbon atom. Also, **14** and **15** were reduced with $LiAlH_4$ in THF and, in both reactions, triol **17** was achieved, with a hydroxymethylene group placed at C-28 [41].

To test the influence, on the induction of apoptosis in cancer cells, of a free carboxyl group at C-28 or its benzyl ester, a series of derivatives (**18**–**21**) were synthesised. Several benzyl derivatives of

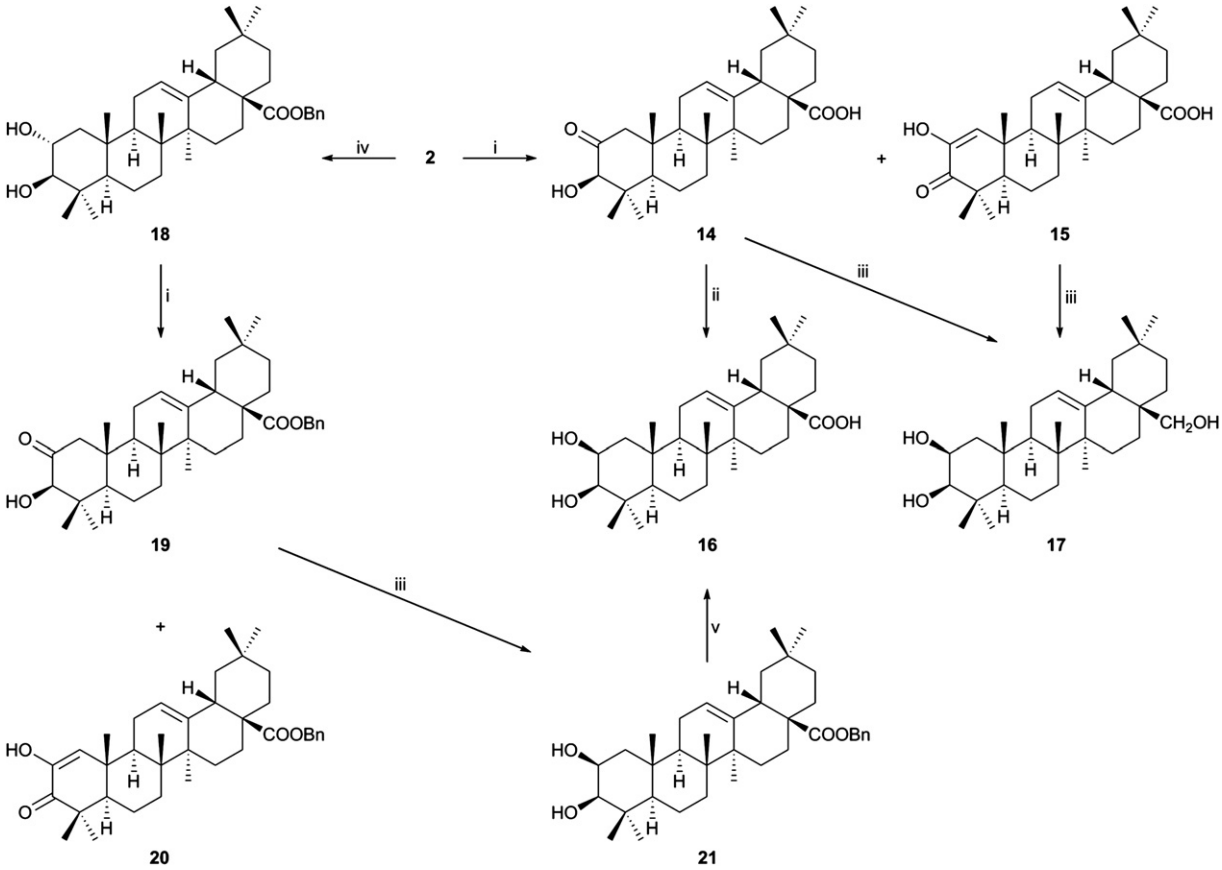


Scheme 1. (i) 1) NaOH, 2) NaCl 4%. (ii) $LiAlH_4$/THF. (iii) 2,2-dimethoxypropane. (iv) TsCl/Py. (v) 1) NaN_3/DCM, 2) *p*-toluenesulfonic acid.



Scheme 2. (i) Ac_2O/Py. (ii) 1) $SOCl_2$/DCM, 2) MeOH/NH_3 (1:1). (iii) KOH/MeOH (1:10). (iv) $SOCl_2$/DCM. (v) $LiAlH_4$/THF.

triterpenoids, previously described, exhibit more potent activity than did their parent compounds [42]. Therefore, MA (**2**) was treated with benzyl chloride and DMF to form 28-benzyl maslinic acid (**18**). Oxidation of **18** with PCC/DCM led to two oxidized compounds **19** and **20**, 28-benzyl derivatives of **14** and **15**, respectively. The reduction of **19** with $LiAlH_4$ in THF gave compound **21** (28-benzyl augustic acid). Finally, deprotection of the benzyl group at C-28 of **21**, was carried out by hydrogenation with H_2/Pd/DCM to get **16**. We easily identified all the compounds of this series (**18**–**21**) by comparing their corresponding ^1H and ^{13}C NMR spectra with those of the analogues with a free carboxyl group at C-28 (**2**, **14**–**16**) (Scheme 3).



Scheme 3. (i) PCC/DCM. (ii) $NaBH_4$/*i*-PrOH. (iii) $LiAlH_4$/THF. (iv) BnCl/K_2CO_3/DMF. (v) H_2/Pd/DCM.

Several compounds (**22**–**40**) [43–47], previously developed by our research group, have also been tested as apoptotic agents. Their structures are shown in Fig. 2.

2.2. Investigation of apoptosis

Apoptosis is a regulated process of cell death that occurs during embryonic development as well as maintenance of tissue homeostasis. Annexin V labelled with FITC can identify and quantify apoptotic cells on a single-cell basis by flow cytometry. Staining cells with propidium iodide and Annexin V-FITC enables the distinction of live, apoptotic, dead and late apoptotic, or necrotic cells [48]. The percentages detected in this test for each cell type, at different concentrations and periods of time, provide information on the mechanism involved in the cell death.

B16-F10 murine melanoma cell lines have been treated with several MA or OA derivatives (**1**–**40**) to test their cytotoxic or apoptotic effects. The results of the flow cytometry for these melanoma cells, which have been treated with a 30 μM solution of these compounds (**1**–**40**) in DMSO for 24 h, are shown in Fig. 3. All the compounds tested, presented very low necrosis (less than 10% in most cases), and there were 10 compounds that exhibited total apoptosis (early and late apoptosis) over 80% (**3**, **10**–**12**, **14**, **15**, **18**–**21**). In most of these compounds, the early apoptosis represented over 90% of total apoptosis. Moreover, the compounds described in previous articles (**22**–**40**), with a great structural variation between them, showed smaller apoptotic effects, and only **25**, **27**, **29**, and **35** had a significant biological activity between 30 and 50% (see Fig. 3).

The compounds with the best results of total apoptosis of the above test, at 30 μM of concentration for 24 h (**3**, **10**–**12**, **14**, **15**, **18**–**21**), were selected to carry out new analyses for 24 h and 48 h at lower concentrations (1, 10, and 20 μM; Figs. 4 and 5). At a concentration of 20 μM for 24 h, compounds **3**, **10**–**12**, **18**–**19** showed a total apoptosis over 70%, whereas for 48 h, almost all the compounds showed this percentage of apoptosis at least. At the lowest concentration (1 μM), the apoptotic activities of certain compounds, for 24 h, were strongly significant: sodium maslinate (**3**, 56.67%), 2,3-diacetoxy-28-cyanide MA (**11**, 68.62%), 28-cyanide MA (**12**, 78.75%), and 28-benzoyl MA (**18**, 87.50%) (Fig. 6). In the compounds with the highest apoptosis activity (**11**, **12** and **18**), at lower concentrations (1 and 10 μM), this apoptosis percentage

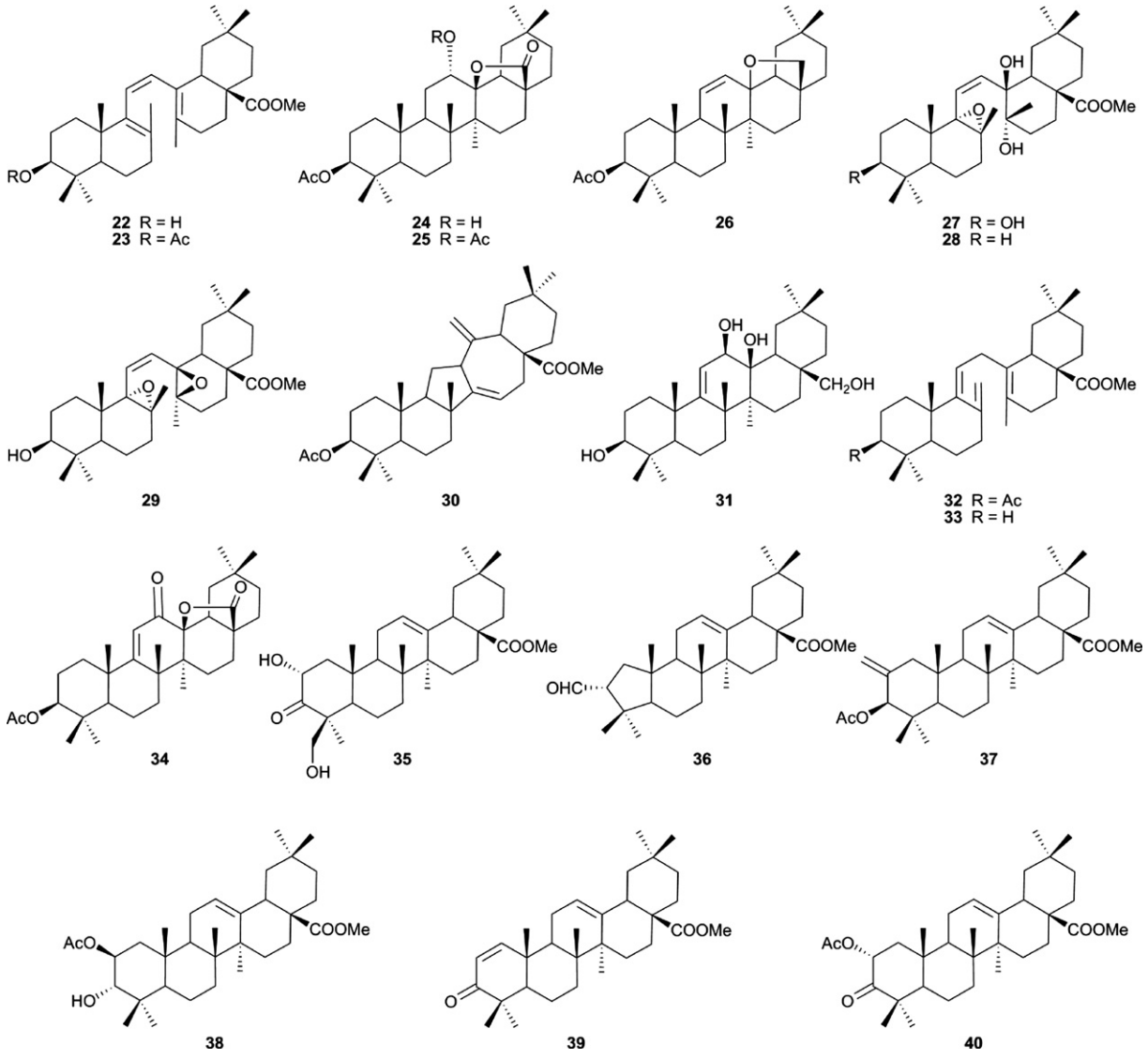


Fig. 2. Compounds **22**–**40**.

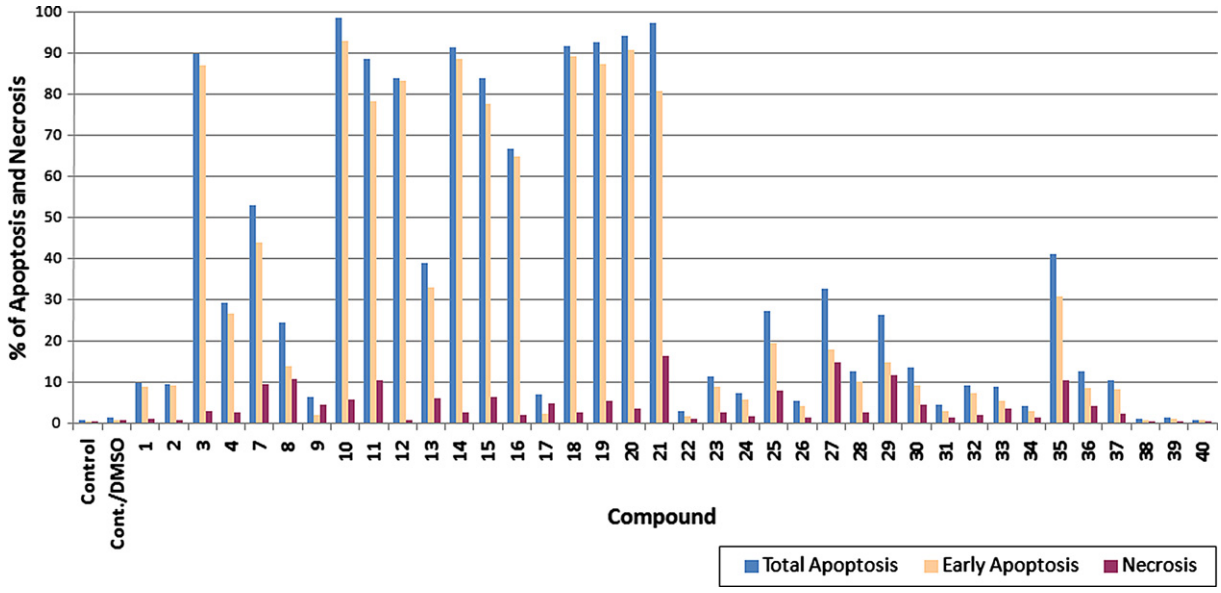


Fig. 3. % of apoptosis (total and early apoptosis) and necrosis at 30 μM of concentration for 24 h.

greatly decreased for 48 h compared to 24 h, possibly due to the fact that the cells that were in apoptosis at 24 h were not present at 48 h, as a consequence of the very process of cell death. Although the structure and activity relationships of these pentacyclic triterpene derivatives are far from clear, it seems that the −COONa, $-CONH_2$, −CN, and −COOBn groups at C-28 enhanced the apoptotic ability of these derivatives as compared to triterpenic acids (−COOH at C-28). Moreover, the necrosis percentage detected with these MA derivatives was insignificant.

3. Conclusions

In conclusion, we have demonstrated that a number of maslinic acid derivatives provide new insight into the anti-carcinogenic action to inhibit B16F10 melanoma. Given that apoptosis induction is arguably the most important process to remove cells that have lost growth control, these maslinic acid derivatives may be considered valuable molecules for use as antiproliferative agents.

4. Experimental

4.1. General

Measurements of NMR spectra (300.13 MHz ^1H and 75.47 MHz ^{13}C) were made in $CDCl_3$ or CD_3OD (which also provided the lock signal) using a BRUKER AM-300 spectrometer. The assignments of ^{13}C chemical shifts were made with the aid of distortionless enhancement by polarization transfer (DEPT) using a flip angle of 135°. Bruker's programs were used for COSY (45°) and C/H and C/C correlation. IR spectra were recorded on a MATTSON SATELLITE FTIR spectrometer. High-resolution mass spectra were made in a MICROMASS AUTOSPEC-Q spectrometer (EBE geometry). Mps were determined using a Kofler (Reichter) apparatus and are uncorrected. Optical rotations were measured on a Perkin−Elmer 241 polarimeter at 25 °C. All reaction solvents and chromatography solvents were distilled prior to use. Commercially available reagents were used without further purification. TLC was carried

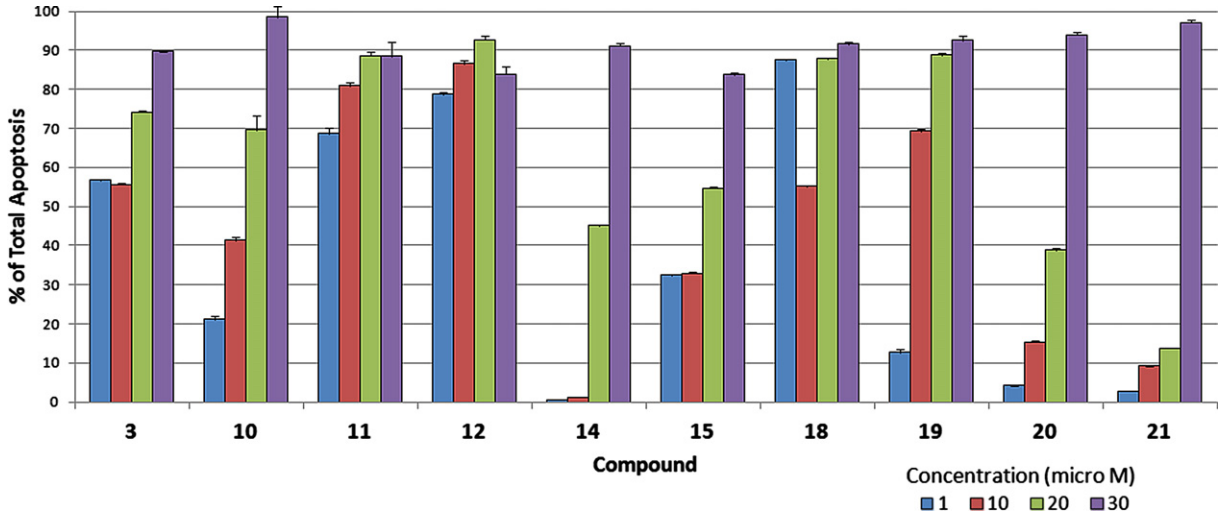


Fig. 4. Total apoptosis *vs.* concentration at 24 h. Data are presented as mean ± SD from two independent experiments.

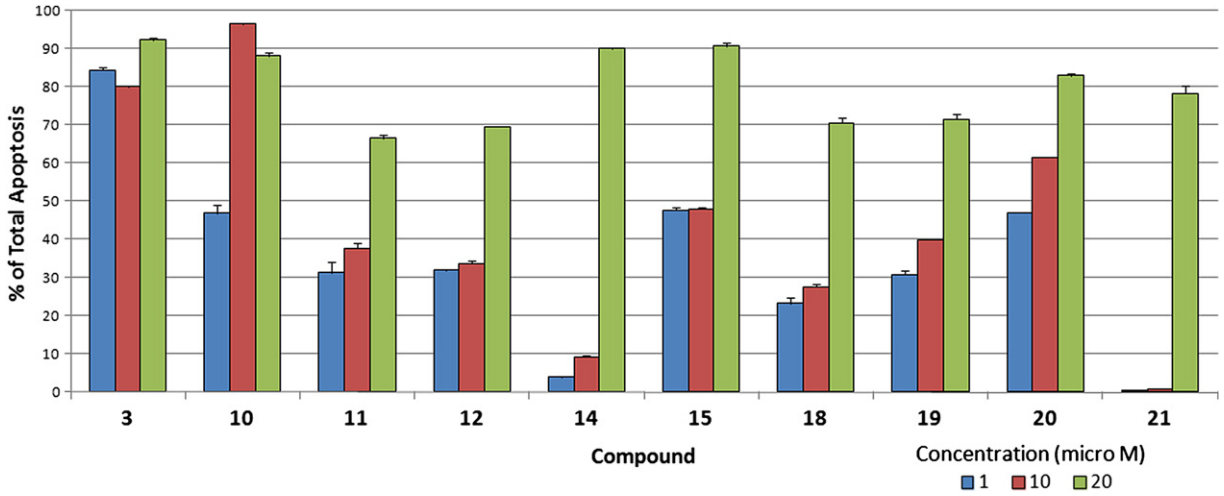


Fig. 5. Total apoptosis *vs.* concentration at 48 h. Data are presented as mean ± SD from two independent experiments.

out on commercially available TLC aluminium sheets, and spots were rendered visible by spraying with H_2SO_4–AcOH, followed by heating to 120 °C, and also visualized under UV at 254 nm. Silica gel (40–60 μm) was used for flash chromatography.

4.2. Synthesis

4.2.1. Sodium maslinate (**3**)

MA (**2**, 200 mg, 0.42 mmol) was added slowly to a stirred solution of NaOH (263 mg) in hot water (20 mL) until its complete dissolution. To facilitate this process, a solution of NaCl (4%) was added. Then, the mixture was cooled and the solid was filtered, washed with a saturated NaCl solution, and with water, to give **3** as a white solid (210 mg, 99%); mp 267–269°; $[\alpha]_D$ +54 (*c* 1 in $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3396, 3001, 2843 and 1710; δ_H ($CDCl_3$) 0.69 (3 H, s, Me), 0.72 (3 H, s, Me), 0.83 (3 H, s, Me), 0.86 (3 H, s, Me), 0.88 (3 H, s, Me), 0.90 (3 H, s, Me), 1.04 (3 H, s, Me), 2.73 (1 H, dd, J = 4.3 and 13.9, H-18), 2.87 (1 H, d, J = 9.1, H-3), 3.59 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2) and 5.03 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 16.3 (Me), 17.2 (Me), 17.5 (Me), 18.2 (C-6), 23.0 (C-16), 23.4 (C-11), 23.7 (Me), 25.7 (Me), 27.6 (C-15), 28.8 (Me), 30.7 (C-20), 32.4 (C-7), 32.5 (C-22), 33.1 (Me), 33.8 (C-21), 37.7 (C-10), 38.8 (C-4), 38.9 (C-8), 41.5 (C-14), 41.7 (C-18), 45.6 (C-17), 46.9 (C-19), 46.9 (C-1), 47.4 (C-9), 54.9 (C-5), 67.1 (C-2), 82.2 (C-3), 119.6 (C-12), 146.1 (C-13) and 181.6 (C-28); HR-LSIMS (*m/z*) 481.3655 ($C_{30}H_{50}O_3Na^+$ requires 481.3658).

4.2.2. Reduction of MA (**2**) with $LiAlH_4$

$LiAlH_4$ (10 mL, 1 M) was added to a solution of MA (**2**, 1 g, 2.1 mmol) in anhydrous THF (30 mL). The reaction was stirred for 2 h at reflux. MeOH was added to destroy the excess reagent, and the solvent was removed under reduced pressure. The residue was purified by column chromatography using DCM/acetone (4:1) to give **4** as a white solid (0.932 g, 93%) [36].

4.2.3. 2,3-Acetonide-2α,3β,28-trihydroxyolean-12-ene (**5**)

A solution of **4** (250 mg, 0.55 mmol) in anhydrous 2,2-dimethoxypropane (10 mL) was stirred for 12 h at room temp. The solvent was removed under reduced pressure and the residue was purified by column chromatography using DCM/acetone (10:1) to give **5** as a white solid (273 mg, 99%); mp 52–54°; $[\alpha]_D$ + 23 (*c* 1, $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3359, 2967, 2899 and 1680; δ_H ($CDCl_3$) 0.85 (3 H, s, Me), 0.86 (3 H, s, Me), 0.86 (3 H, s, Me), 0.93 (3 H, s, Me), 1.01 (3 H, s, Me), 1.03 (3 H, s, Me), 1.14 (3 H, s, Me), 1.38 (3 H, s, Me acetonide group), 1.40 (3 H, s, Me acetonide group), 2.71 (1 H, dd, J = 4.3 and 13.9, H-18), 3.01 (1 H, d, J = 9.1, H-3), 3.19 (1 H, d, J = 12.5, H_B-28), 3.52 (1 H, d, J = 12.5, H_A-28), 3.66 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2) and 5.17 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 16.4 (Me), 17.3 (Me), 17.5 (Me), 18.0 (C-6), 22.2 (C-16), 23.8 (Me), 24.0 (C-11), 25.8 (C-15), 26,2 (Me), 27.1 (Me acetonide group), 27.4 (Me), 27.4 (Me acetonide group), 28.7 (C-20), 31.3 (C-22), 32.9 (C-7), 33.4 (Me), 34.3 (C-21), 37.2 (C-10), 37.6 (C-4), 40.2 (C-8), 40.4 (C-14), 42.0 (C-17), 42.2 (C-1), 42.5 (C-18), 46.6 (C-19), 48.1 (C-9), 56.3 (C-5), 69.9 (C-28), 72.7 (C-2), 88.8 (C-3), 108.4 (C acetonide group), 122.3 (C-12) and 144.6 (C-13); HR-LSIMS (*m/z*) 497.4077 ($C_{33}H_{54}O_3^+$ requires 497.4073).

4.2.4. 2,3-Acetonide-2α,3β-dihydroxy-28-tosylolean-12-ene (**6**)

Tosyl chloride (300 mg, 0.46 mmol) was added to a solution of **5** (500 mg, 0.8 mmol) in anhydrous pyridine (10 mL). The reaction was stirred for 24 h at room temp. The mixture was extracted with DCM and the organic layer dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by column chromatography using hexane/ethyl acetate (8:1) to give **6** as a white solid in quantitative yield (663 mg); mp 101–103°; $[\alpha]_D$ +27 (*c* 1, $CHCl_3$); IR ν_{max}(KBr)/cm^{-1} 3379, 2895, 2845 and 1700; δ_H ($CDCl_3$) 0.81 (3 H, s, Me), 0.84 (3 H, s, Me), 0.86 (3 H, s, Me), 0.86 (3 H, s, Me), 0.97 (3 H, s, Me), 1.02 (3 H, s, M), 1.08 (3 H, s, Me), 1.38 (3 H s, Me acetonide group), 1.39 (3 H, s, Me acetonide group), 2.42 (3 H, s, Me tosyl group), 2.71 (1 H, dd, J = 4.3 and 13.9, H-18), 3.00 (1 H, d, J = 9.1, H-3), 3.50 (1 H, d, J = 12.5, H_A-28), 3.66 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2), 3.87 (1 H, d, J = 12.5, H_B-28), 5.05 (1 H, dd, J = 3.5 and 3.5, H-12), 7.30 (2 H, d, J = 9.0, H tosyl group) and 7.74 (2 H, d, J = 9.0, H tosyl group); δ_C ($CDCl_3$) 16.4 (Me), 16.7 (Me), 17.4 (Me), 17.9 (C-6), 20.6 (Me tosyl group), 21.8 (Me), 22.8 (C-16), 23.8 (Me), 23.9 (C-11), 25.7 (C-15), 26,2 (Me), 27.3 (Me acetonide group), 28.7 (Me acetonide group), 31.0 (C-20), 31.8 (C-22), 32.7 (C-7), 33.2 (Me), 33.9 (C-21), 35.5 (C-10), 37.5 (C-4), 40.0 (C-8), 40.2 (C-14), 41.7 (C-17), 42.1 (C-18), 42.2 (C-1), 46.3 (C-19), 47.9 (C-9), 56.2 (C-5), 72.6 (C-2), 76.8 (C-28), 88.8 (C-3), 108.4 (C acetonide group), 123.1 (C-12), 128.2 (C tosyl group), 129.9 (C tosyl group), 133.3 (C tosyl group), 143.3 (C tosyl group) and 144.6 (C-13); HR-LSIMS (*m/z*) 651.4157 ($C_{40}H_{60}O_5S^+$ requires 651.4161).

4.2.5. 2α,3β-Dihydroxyolean-12-en-28-azide (**7**)

NaN_3 (90 mg) was added to a solution of **6** (300 mg, 0.48 mmol) in DCM (25 mL). The reaction was stirred for 4 h at room temp. The

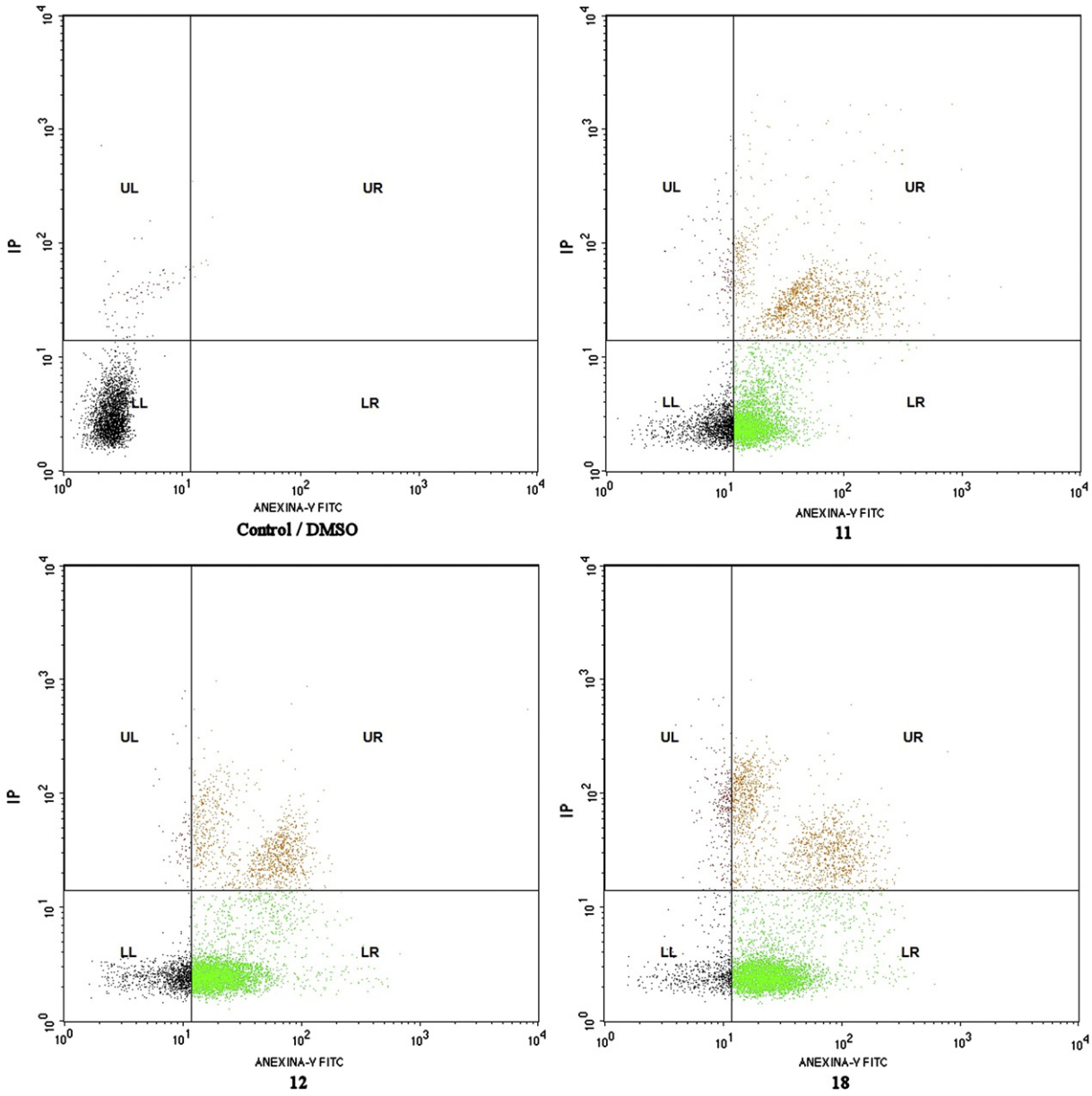


Fig. 6. Flow cytometry for control/DMSO and compounds **11**, **12**, and **18** at a concentration of 1 μM for 24 h. Low left quadrant (LL, black): live cells. Low right quadrant (LR, green): early apoptotic cells. Upper right quadrant (UR, orange): late apoptotic and dead cells. Upper left quadrant (UL, fuchsia): necrotic cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mixture was extracted with DCM, *p*-toluenesulfonic acid (20 mg) was added, and the reaction was stirred for 30 min at room temp. The mixture was extracted with DCM, and the organic layer was dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by column chromatography using DCM/acetone (2:1) to give **7** as a white solid (77 mg, 93%); mp 89–91°; $[\alpha]_D$ +62 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3392, 2950, 2883 and 1664; δ_H ($CDCl_3$) 0.85 (3 H, s, Me), 0.87 (3 H, s, Me), 0.93 (3 H, s, Me), 0.95 (3 H, s, Me), 0.99 (3 H, s, Me), 1.03 (3 H, s, Me), 1.16 (3 H, s, Me), 2.71 (1 H, dd, $J = 4.3$ and 13.9, H-18), 3.13 (1H, d, $J = 9.0$, H-3), 3.34 (1 H, d, $J = 9.0$, H_A-28), 3.68 (1 H, d, $J = 9.0$, H_B-28), 3.80 (1 H, ddd, $J = 4.4$, 9.0 and 11.2, H-2) and 5.19 (1 H, dd, $J = 3.5$ and 3.5, H-12); δ_C ($CDCl_3$) 17.0 (Me), 17.1 (Me), 17.8 (Me), 18.6 (C-6), 22.2 (C-16), 22.2 (C-11), 25.7 (Me), 25.8 (C-15), 26.2 (Me), 28.8 (Me), 29.5 (C-20), 31.3 (C-22), 32.7 (C-7), 33.4 (Me), 34.3 (C-21), 37.2 (C-10), 38.4 (C-4), 39.4 (C-8), 40.1 (C-14), 42.0 (C-17), 42.6 (C-18), 46.7 (C-1), 46.8 (C-19), 47.7 (C-9), 55.5 (C-5), 69.2 (C-2), 69.9 (C-28), 84.1 (C-3), 122.4 (C-12) and 144.5 (C-13); HR-LSIMS (*m/z*) 482.3820 ($C_{30}H_{49}O_2N_3^+$ requires 482.3825).

4.2.6. Acetylation of MA (*2*) with Ac_2O

Ac_2O (2 mL) was added slowly to a solution of MA (**2**, 1.4 g, 0.294 mol) in pyridine (10 mL). The reaction was stirred for 2 h at reflux. Cold water was added to the mixture and, after this was extracted with DCM, the organic layer was dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by column chromatography using DCM/acetone (10:1) to give **8** as a white solid (924 mg, 70%) [39].

4.2.7. 2α,3β-Diacetoxyolean-12-en-28-amide (*9*)

$SOCl_2$ (2 mL) was added to a solution of **8** (500 mg, 0.87 mmol) in anhydrous DCM (20 mL). The reaction was stirred for 2 h at

reflux. The solvent was removed under reduced pressure and the residue dissolved in MeOH. Then, a solution of MeOH/NH_3 (1:1) was added dropwise up to the cessation of the bubbling. The mixture was stirred overnight at room temp. The mixture was extracted with DCM and the organic layer dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the residue purified by column chromatography using hexane/ethyl acetate (2:1) to give **9** as a white solid (109 mg, 55%); mp 267–269°; $[\alpha]_D$ +54 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3399, 2912, 2855 and 1708; δ_H ($CDCl_3$) 0.84 (3 H, s, Me), 0.85 (3 H, s, Me), 0.91 (3 H, s, Me), 0.93 (3 H, s, Me), 1.07 (3 H, s, Me), 1.10 (3 H, s, Me), 1.18 (3 H, s, Me), 1.94 (3 H, s, Me acetyl group), 2.02 (3 H, s, Me acetyl group), 2.49 (1 H, dd, J = 4.3 and 13.9, H-18), 4.71 (1H, d, J = 9.1, H-3), 5.04 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2) and 5.90 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 17.8 (Me), 17.1 (Me), 17.6 (Me), 18.3 (C-6), 21.1 (Me acetyl group), 21.3 (Me acetyl group), 23.5 (Me), 23.9 (C-11), 23.9 (C-16), 25.9 (Me), 27.4 (C-15), 28.6 (Me), 30.9 (C-20), 32.4 (C-22), 32.6 (C-7), 33.2 (Me), 34.2 (C-21), 38.3 (C-10), 39.4 (C-4), 39.5 (C-8), 42.2 (C-14), 42.6 (C-18), 44.1 (C-19), 46.5 (C-17), 46.8 (C-1), 47.64 (C-9), 55.0 (C-5), 70.2 (C-2), 80.2 (C-3), 122.7 (C-12), 145.0 (C-13), 170.7 (C=O acetyl group), 171.0 (C=O acetyl group) and 182.2 (C-28); HR-LSIMS (*m/z*) 556.3998 ($C_{34}H_{54}O_5N^+$ requires 556.4002).

*4.2.8. 2α,3β-Dihydroxyolean-12-en-28-amide (**10**)*

A solution of KOH/MeOH (1:10, 5 mL) was added to a solution of **9** (100 mg, 0.18 mmol) in anhydrous THF (5 mL). The reaction was stirred for 30 min at room temp. Then, cold water was added dropwise up to the cessation of the bubbling. The mixture was extracted with DCM. The organic layer was dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by column chromatography using DCM/acetone (2:1) to give **10** as a white solid (76 mg, 90%); mp 160–162°; $[\alpha]_D$ +60 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3401, 2940, 2890 and 1698; δ_H ($CDCl_3$) 0.86 (3 H, s, Me), 0.86 (3 H, s, Me), 0.95 (3 H, s, Me), 0.95 (3 H, s, Me), 1.02 (3 H, s, Me), 1.06 (3 H, s, Me), 1.20 (3 H, s, Me), 2.56 (1 H, dd, J = 4.3 and 13.9, H-18), 3.03 (1H, d, J = 9.1, H-3), 3.71 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2) and 5.90 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 16.9 (Me), 17.0 (Me), 17.3 (Me), 18.5 (C-6), 23.8 (Me), 23.8 (C-16), 24.0 (C-11), 26.0 (Me), 27.5 (C-15), 27.8 (Me), 30.9 (C-20), 32.6 (C-22), 32.7 (C-7), 33.2 (Me), 34.3 (C-21), 38.5 (C-10), 39.4 (C-4), 39.5 (C-8), 42.4 (C-14), 42.8 (C-18), 46.6 (C-19), 46.7 (C-17), 46.9 (C-1), 47.8 (C-9), 55.5 (C-5), 69.1 (C-2), 84.1 (C-3), 122.9 (C-12), 145.2 (C-13) and 181.5 (C-28); HR-LSIMS (*m/z*) 494.3592 ($C_{30}H_{49}O_3Na^+$ requires 494.3610).

*4.2.9. 2α,3β-Diacetoxyolean-12-en-28-nitrile (**11**)*

$SOCl_2$ (2 mL) was added to a solution of **9** (200 mg, 0.36 mmol) in anhydrous DCM (10 mL). The reaction was stirred for 3 h at room temp. Then, cold water was added and the mixture was extracted with DCM. The organic layer was dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by column chromatography using DCM/acetone (10:1) to give **11** as a white solid (83 mg, 43%); mp 144–146°; $[\alpha]_D$ +34 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 2929, 2360, 1741 and 1250; δ_H ($CDCl_3$) 0.89 (3 H, s, Me), 0.90 (3 H, s, Me), 0.92 (3 H, s, Me), 1.01 (3 H, s, Me), 1.07 (3 H, s, Me), 1.11 (3 H, s, Me), 1.23 (3 H, s, Me), 1.95 (3 H, s, Me acetyl group), 2.03 (3 H, s, Me acetyl group), 2.56 (1 H, dd, J = 4.3 and 13.9, H-18), 4.73 (1H, d, J = 9.1, H-3), 5.07 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2) and 5.34 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 16.7 (Me), 17.4 (Me), 17.9 (Me), 18.4 (C-6), 21.1 (Me acetyl group), 21.3 (Me acetyl group), 23.6 (Me), 23.7 (C-16), 24.2 (C-11), 25.7 (Me), 28.2 (C-15), 28.7 (Me), 30.8 (C-20), 32.4 (C-22), 32.6 (C-7), 33.2 (Me), 34.2 (C-21), 38.2 (C-10), 38.3 (C-4), 39.6 (C-8), 39.7 (C-14), 42.6 (C-18), 44.1 (C-19), 44.2 (C-1), 46.8 (C-17), 47.7 (C-9), 55.1 (C-5), 70.2 (C-2), 80.8 (C-3), 124.4 (C-12), 125.7 (C-28), 141.9 (C-13), 170.7 (C=O acetyl group) and 171.0 (C=O acetyl group); HR-LSIMS (*m/z*) 560.3710 ($C_{34}H_{51}O_4NNa^+$ requires 560.3716).

*4.2.10. 2α,3β-Dihydroxyolean-12-en-28-nitrile (**12**)*

A solution of KOH/MeOH (1:10, 5 mL) was added to a solution of **11** (100 mg, 0.18 mmol) in anhydrous THF (5 mL). Proceeding as described above for compound **10**, compound **12** was obtained as a white solid (77 mg, 90%); mp 105–107°; $[\alpha]_D$ +40 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 2955, 2383, 1755 and 1270; δ_H ($CDCl_3$) 0.82 (3 H, s, Me), 0.89 (3 H, s, Me), 0.92 (3 H, s, Me), 1.00 (3 H, s, Me), 1.01 (3 H, s, Me), 1.02 (3 H, s, Me), 1.12 (3 H, s, Me), 2.56 (1 H, dd, J = 4.3 and 13.9, H-18), 2.99 (1H, d, J = 9.1, H-3), 3.69 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2) and 5.35 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 16.9 (Me), 17.0 (Me), 17.5 (Me), 18.6 (C-6), 23.6 (Me), 23.7 (C-16), 24.3 (C-11), 25.8 (Me), 28.2 (C-15), 28.9 (Me), 30.8 (C-20), 32.5 (C-22), 33.1 (C-7), 33.2 (Me), 33.2 (C-21), 38.2 (C-10), 38.5 (C-8), 39.4 (C-4), 39.8 (C-14), 42.1 (C-17), 44.1 (C-18), 45.0 (C-19), 46.7 (C-1), 47.8 (C-9), 55.5 (C-5), 69.1 (C-2), 84.1 (C-3), 124.7 (C-12), 125.8 (C-28) and 141.9 (C-13); HR-LSIMS (*m/z*) 476.3513 ($C_{30}H_{47}O_2NNa^+$ requires 476.3504).

*4.2.11. 2α,3β-Dihydroxyolean-12-en-28-amine (**13**)*

$LiAlH_4$ (1 mL, 1 M) was added to a solution of **11** (100 mg, 0.2 mmol) in anhydrous THF (3 mL). The reaction was stirred for 2 h at reflux. The mixture was extracted with DCM and the organic layer dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue purified by column chromatography using DCM/acetone (2:1) to give **13** as a white solid (75 mg, 80%); mp 135–137°; $[\alpha]_D$ +63 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 2933, 2342, 1761 and 1245; δ_H ($CDCl_3$) 0.85 (3 H, s, Me), 0.90 (3 H, s, Me), 0.91 (3 H, s, Me), 0.96 (3 H, s, Me), 1.03 (3 H, s, Me), 1.06 (3 H, s, Me), 1.19 (3 H, s, Me), 2.58 (1 H, dd, J = 4.3 and 13.9, H-18), 3.03 (1H, d, J = 9.1, H-3), 3.23 (1 H, d, J = 12.0, H_A-28), 3.57 (1 H, d, J = 12.0, H_B-28), 3.71 (1 H, ddd, J = 4.4, 9.1 and 11.2, H-2) and 5.22 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 17.0 (Me), 17.0 (Me), 17.1 (Me), 18.6 (C-6), 22.2 (C-16), 23.8 (C-11), 23.9 (C-15), 23.9 (Me), 25.7 (Me), 28.8 (Me), 29.5 (C-20), 31.3 (C-22), 32.7 (C-7), 33.4 (Me), 34.3 (C-21), 37.2 (C-10), 38.4 (C-8), 39.4 (C-4), 40.1 (C-14), 42.0 (C-17), 42.6 (C-18), 46.7 (C-1), 46.7 (C-19), 47.8 (C-9), 55.5 (C-5), 69.8 (C-2), 69.9 (C-28), 84.1 (C-3), 122.4 (C-12) and 144.5 (C-13); HR-LSIMS (*m/z*) 456.3913 ($C_{30}H_{51}O_2N^+$ requires 456.3920).

*4.2.12. Oxidation of MA (**2**) with PCC*

PCC (90 mg, 5.4 mmol) was added to a solution of **2** (200 mg, 0.36 mmol) in dry DCM (10 mL). The mixture was stirred for 2 h at room temp. The mixture was extracted with a solution of hexane/diethyl ether (1:1) and the organic layer dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by column chromatography using hexane/ethyl acetate (2:1) to give **14** as a white solid (67 mg, 40%); mp 164–166°; $[\alpha]_D$ +85 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3400, 2953, 2868 and 1639; δ_H ($CDCl_3$) 0.71 (3 H, s, Me), 0.78 (3 H, s, Me), 0.90 (3 H, s, Me), 0.94 (3 H, s, Me), 0.96 (3 H, s, Me), 1.22 (3 H, s, Me), 1.22 (3 H, s, Me), 2.87 (1 H, dd, J = 4.3 and 13.9, H-18), 3.92 (1 H, s, H-3) and 5.32 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C ($CDCl_3$) 16.4 (Me), 16.8 (Me), 16.9 (Me), 18.7 (C-6), 23.1 (C-16), 23.6 (C-11), 23.8 (Me), 26.1 (Me), 27.6 (C-15), 29.6 (Me), 30.9 (C-20), 32.6 (C-7), 32.6 (C-22), 33.2 (Me), 34.0 (C-21), 39.9 (C-8), 41.2 (C-18), 42.0 (C-14), 43.9 (C-10), 46.0 (C-4), 46.1 (C-19), 46.7 (C-17), 47.9 (C-9), 53.3 (C-1), 54.7 (C-5), 83.1 (C-3), 122.1 (C-12), 144.0 (C-13), 183.2 (C-28) and 211.2 (C-2); HR-LSIMS (*m/z*) 469.3390 ($C_{30}H_{46}O_4^+$ requires 469.3396); and **15**, also as a white solid (67 mg, 40%) [39].

*4.2.13. Augustic acid (2β,3β-dihydroxyolean-12-en-28-oic acid) (**16**)*

$NaBH_4$ (40 mg) was added to a solution of **14** (100 mg, 0.21 mmol) in *i*-PrOH (5 mL). The reaction was stirred for 2 h at room temp. MeOH was added to destroy the excess reagent, and the solvent removed under reduced pressure. The residue was purified by column chromatography using DCM/acetone (4:1) to give **16** as a white solid (95.2 mg, 95%); mp 265–267°; $[\alpha]_D$ +83 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3380, 2912, 2890 and 1676; δ_H (CD_3OD) 0.62 (3 H, s, Me), 0.69 (3 H, s, Me), 0.76 (3 H, s, Me), 0.94 (3 H, s, Me), 0.99 (3 H, s, Me), 1.04 (3 H, s, Me), 1.19 (3 H, s, Me), 2.99 (1 H, dd, J = 4.3 and 13.9, H-18), 3.11 (1 H, d, J = 3.1, H-3), 4.08 (1 H, ddd, J = 3.1, 3.1 and 4.4, H-2) and 5.19 (1 H, dd, J = 3.5 and 3.5, H-12); δ_C (CD_3OD) 16.7 (Me), 17.6 (Me), 18.3 (Me), 18.8 (C-6), 23.2 (C-16), 23.9 (Me), 24.1 (C-11), 26.4 (Me), 28.7 (C-15), 30.4 (Me), 30.7 (C-20), 33.4 (Me), 33.5 (C-22), 33.7 (C-7), 34.4 (C-21), 37.8 (C-8), 39.2 (C-4), 40.3 (C-10), 42.2 (C-18), 42.8 (C-14), 45.0 (C-1), 46.5 (C-17), 46.6 (C-19), 48.7 (C-9), 56.4 (C-5), 71.9 (C-2), 78.8 (C-3), 123.1 (C-12), 145.3 (C-13) and 180.6 (C-28); HR-LSIMS (*m/z*) 495.3455 ($C_{30}H_{48}O_4Na^+$ requires 495.3450).

*4.2.14. 2β,3β,28-Trihydroxyolean-12-ene (**17**)*

$LiAlH_4$ (1 mL, 1 M) was added to a solution of **14** (100 mg, 0.21 mmol) in anhydrous THF (5 mL). Proceeding as described above for the reduction of MA (**2**), compound **17** was obtained as a white solid (93.2 mg, 93%). The compound **17** also was synthesised from **15** under the same reaction conditions (92.1 mg, 91%) [41].

*4.2.15. Benzyl maslinate (**18**)*

BnCl was added to a solution of MA (**2**, 1 g, 2 mmol) in DMF (8 mL) with K_2CO_3 (0.61 g). The reaction was stirred for 4 h at 55 °C. The mixture was washed with water and extracted with DCM, and the organic layer dried with anhydrous Na_2SO_4. The solvent was removed under reduced pressure, the residue was purified by column chromatography using DCM/acetone (10:1) to give **18** as a white solid (690 mg, 81%); mp 226–228°; $[\alpha]_D$ +12 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3396, 2925, 2843 and 1704; δ_H ($CDCl_3$) 0.50 (3 H, s, Me), 0.72 (3 H, s, Me), 0.80 (3 H, s, Me), 0.82 (3 H, s, Me), 0.85 (3 H, s, Me), 0.92 (3 H, s, Me), 1.03 (3 H, s, Me), 2.82 (1 H, dd, J = 3.5 and 12.2, H-18), 2.89 (1 H, d, J = 9.1, H-3), 3.59 (1 H, ddd, J = 9.1 and 12.1, H-2), 4.97 (2 H, AB system, J = 12.0, H benzyl group), 5.21 (1 H, dd, J = 3.5 and 3.5, H-12) and 7.48 (5 H, m, H benzyl group); δ_C ($CDCl_3$) 16.9 (Me), 17.2 (Me), 17.3 (Me), 18.7 (C-6), 23.3 (C-16), 23.8 (C-11), 24.0 (Me), 27.9 (C-15), 29.02 (Me), 30.9 (C-20), 32.7 (C-22), 32.9 (C-7), 33.5 (Me), 34.0 (C-21), 38.4 (C-4), 39.2 (C-10), 39.5 (C-8), 41.5 (C-14), 41.9 (C-18), 46.0 (C-17), 46.2 (C-1), 46.8 (C-19), 47.9 (C-9), 55.6 (C-5), 66.3 (benzyl-C), 69.2 (C-2), 89.0 (C-3), 122.5 (C-12), 128.0 (C benzyl group), 128.1 (C benzyl group), 128.5 (C benzyl group), 136.5 (C benzyl group), 143.9 (C-13) and 177.2 (C-28); HR-LSIMS (*m/z*) 585.3910 ($C_{37}H_{54}O_4Na^+$ requires 585.3920).

*4.2.16. Oxidation of **18** with PCC*

PCC (90 mg, 5.4 mmol) was added to a solution of **18** (200 mg, 0.36 mmol) in DCM (10 mL). Proceeding as described above for the oxidation of MA (**2**), compound **19** was obtained as a white solid (77 mg, 40%); mp 107–109°; $[\alpha]_D$ +52 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3366, 2963, 2832 and 1720; δ_H ($CDCl_3$) 0.50 (3 H, s, Me), 0.61 (3 H, s, Me), 0.76 (3 H, s, Me), 0.82 (3 H, s, Me), 0.84 (3 H, s, Me), 1.09 (3 H, s, Me), 1.10 (3 H, s, Me), 3.09 (1 H, dd, J = 3.1 and 12.2, H-18), 4.04 (1 H, s, H-3), 5.21 (2 H, AB system, J = 12.0, H benzyl group), 5.21 (1 H, dd, J = 3.5 and 3.5, H-12) and 7.48 (5 H, m, H benzyl group); δ_C ($CDCl_3$) 16.4 (Me), 16.7 (Me), 16.8 (Me), 18.7 (C-6), 23.2 (C-16), 23.5 (C-11), 23.8 (Me), 26.0 (Me), 27.8 (C-15), 29.6 (Me), 30.9 (C-20), 32.5 (C-22), 32.5 (C-7), 33.3 (Me), 34.1 (C-21), 39.9 (C-8), 41.6 (C-18), 42.0 (C-14), 43.9 (C-10), 45.9 (C-4), 46.1 (C-19), 46.9 (C-17), 46.8 (C-9), 53.3 (C-1), 54.7 (C-5), 66.17 (C benzyl group), 83.2 (C-3), 121.9 (C-12), 128.1 (C benzyl group), 128.2 (C benzyl group), 128.6 (C benzyl group), 136.6 (C benzyl group), 144.1 (C-13), 177.5 (C-28) and 211.2 (C-2); HR-LSIMS (*m/z*) 561.3943 ($C_{37}H_{53}O_4^+$ requires 561.3944); and also **20** as a white solid (77 mg, 40%); mp 199–201°; $[\alpha]_D$ +47 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3391, 2925, 2897 and 1700; δ_H ($CDCl_3$) 0.57 (3 H, s, Me), 0.81 (3 H, s, Me), 0.84 (3 H, s, Me), 1.02 (3 H, s, Me), 1.03 (3 H, s, Me), 1.10 (3 H, s, Me), 1.12 (3 H, s, Me), 2.94 (1 H, dd, J = 3.1 and 15.0, H-18), 5.10 (2 H, AB system, J = 12.0, H benzyl group), 5.25 (1 H, dd, J = 3.5 and 3.5, H-12), 6.24 (1 H s, H-1) and 7.38 (5 H, m, H benzyl group); δ_C ($CDCl_3$) 17.5 (Me), 18.9 (C-6), 19.8 (Me), 22.0 (Me), 23.3 (C-16), 23,7 (11), 23.8 (Me), 26.0 (Me), 27.7 (C-15), 27.9 (Me), 30.9 (C-20), 32.6 (C-22), 32.8 (C-7), 33.3 (Me), 33.6 (C-8), 34.2 (C-21), 40.2 (C-14), 41.7 (C-18), 42.3 (C-10), 43.2 (C-9), 44.1 (C-4), 46.0 (C-19), 47.0 (C-17), 54.1 (C-5), 66.3 (C benzyl group), 122.3 (C-12), 128.2 (C benzyl group), 128.2 (C benzyl group), 128.5 (C-1), 128.6 (C benzyl group), 136.5 (C benzyl group), 143.9 (C-2), 144.3 (C-13), 177.6 (C-28) and 201.3 (C-3); HR-LSIMS (*m/z*) 559.3765 ($C_{37}H_{51}O_4^+$ requires 559.3787).

*4.2.17. Benzyl augustate (**21**)*

$LiAlH_4$ (1 mL, 1 M) was added to a solution of **19** (100 mg, 0.18 mmol) in anhydrous THF (10 mL). Proceeding as described above for the reduction of MA (**2**), compound **21** was obtained as a white solid (90.1 mg, 91%); mp 221–223°; $[\alpha]_D$ +64 (*c* 1, $CHCl_3$:MeOH, 2:1); IR ν_{max}(KBr)/cm^{-1} 3371, 2940, 2891 and 1668; δ_H ($CDCl_3$) 0.60 (3 H, s, Me), 0.87 (3 H, s, Me), 0.90 (3 H, s, Me), 0.98 (3 H, s, Me), 0.98 (3 H, s, Me), 1.10 (3 H, s, Me), 1.18 (3 H, s, Me), 2.83 (1 H, dd, J = 3.1 and 12.0, H-18), 3.10 (1 H, d, J = 3.0, H-3), 3.98 (1 H, ddd, J = 3.0, 4.4 and 4.4, H-2), 5.00 (2 H, AB system, J = 12.0, H benzyl group), 5.28 (1 H, dd, J = 3.5 and 3.5, H-12) and 7.48 (5 H, m, H benzyl group); δ_C ($CDCl_3$) 16.5 (Me), 17.1 (Me), 17.5 (Me), 18.3 (C-6), 23.3 (C-16), 23.7 (C-11), 23.8 (Me), 26.1 (Me), 27.7 (C-15), 29.9 (Me), 30.9 (C-20), 32.6 (C-22), 32.9 (C-7), 33.3 (Me), 34.08 (C-21), 36.9 (C-8), 38.3 (C-4), 39.6 (C-10), 41.6 (C-18), 42.0 (C-14), 44.3 (C-1), 46.1 (C-19), 47.0 (C-17), 48.3 (C-9), 55.43 (C-5), 66.1 (C benzyl group), 71.3 (C-2), 78.7 (C-3), 122.8 (C-12), 128.1 (C benzyl group), 128.2 (C benzyl group), 128.6 (C benzyl group), 136.6 (C benzyl group), 143.9 (C-13) and 177.6 (C-28); HR-LSIMS (*m/z*) 561.3937 ($C_{37}H_{53}O_4^+$ requires 561.3937).

*4.2.18. Hydrogenation of **21***

Palladium was added in catalytic amount to a solution of **21** (200 mg, 0.39 mmol) in anhydrous DCM (20 mL). The reaction was stirred for 2 h at 4 atm hydrogen pressure. Then, the mixture was filtered and the solvent removed under reduced pressure. The residue was purified by column chromatography using DCM/acetone (2:1) to give **16** as a white solid (165 mg, 90%).

4.3. Apoptosis tests

B16-F10 murine melanoma cell lines (ATCC N°: CRL-6475) were provided by the Scientific Instrumentation Centre of University of Granada. B16-F10 murine melanoma cells were grown in DMEM medium containing L-glutamine (4 mM), sodium bicarbonate (1.5 g/L), sodium pyruvate (1 mM), glucose (4.5 g/L), and foetal bovine serum (10%). Confluents B16-F10 melanoma cells in DMEM medium with foetal bovine serum, 10%, were trypsinised and put on 6-well culture dishes (300,000 cells per well). After 24 h, the cells were treated with the compounds **1**–**40** (by duplicate with compounds **3**, **10**–**12**, **14**, **15**, **18**–**21**) in DMSO at different concentrations for 24 or 48 h. As a control, B16F10 cells were incubated in the presence of the vehicle (DMSO) or in medium alone.

Afterwards, the cells were detached with EDTA-Trypsin (0.5 mg/mL and 0.2 mg/mL, respectively), washed twice with temperate phosphate-buffered saline (PBS), and resuspended in 1× Annexin-binding buffer at a concentration 1×10^6 cells/mL. 5 μL of the Annexin V-FITC and 5 μL of propidium iodide were added to 100 μL of each sample. The cells were incubated at room temperature for 15 min in darkness. After this incubation period, 400 μL of 1× Annexin-binding buffer were added and the samples analysed by flow cytometry within 1 h.

Samples were analysed on a Becton Dickinson FACSVantage flow cytometer with a filter BP530 ± 30 nm in FL1 and a filter BP585 ± 70 nm in FL2 to collect the fluorescein and propidium iodide signals, respectively. The acquisition process was at 500 cells/sec up to completing 10,000 cells with CELLQuest Software (Becton Dickinson).

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