European Journal of Medicinal Chemistry 46 (2011) 1356–1366

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

A comparative study of proapoptotic potential of cyano analogues of boswellic acid and 11-keto-boswellic acid

Rajbir Kaur, Sheema Khan, Renu Chib, Tandeep Kaur, Parduman Raj Sharma, Jaswant Singh, Bhahwal Ali Shah*, Subhash Chandra Taneja*

Indian Institute of Integrative Medicine (CSIR), Canal Road, Jammu Tawi-180001, India

ARTICLE INFO

Article history:
Received 16 September 2010
Accepted 26 January 2011
Available online 3 February 2011

Keywords:
Triterpenoids
Boswellic acids
Anti-cancer
Cytotoxicity
Apoptosis

ABSTRACT

Semi-synthetic analogues of β-boswellic acid (BA) and 11-keto-β-boswellic acid (KBA) were comparatively evaluated for *in vitro* cytotoxicity against human myeloid leukaemia (HL-60) and human cervical carcinoma (HeLa) cells. 2-Cyano analogues of both the triterpenes were observed to have significant cytotoxicity against both the cells, displaying cytotoxicity in HL-60 cells at low concentrations. Further investigations suggested the proapoptotic potential associated with the two molecules to induce cytotoxicity in HL-60 cells, where one of them showed early proapoptotic effect as evidenced by several biological end-points of the apoptosis such as annexinV binding, DNA fragmentation and increase in sub-G0 DNA fraction and apoptotic bodies formation (Hoechst 33258 staining and SEM studies).

1. Introduction

Dysregulation of cell death mechanisms is involved in the pathogenesis of an increasing number of diseases like cancer. An alteration in thresholds for apoptosis is becoming a prominent concept, as we move along in our quest to understand and ultimately treat cancer. Because the dysregulation of apoptosis is the hallmark of cancer cells, the activation of apoptosis is a common mechanism of malignant cell killing by anti-cancer agents [1]. Natural products and the molecules based on natural product scaffolds being the proven source of therapeutic agents have increasingly attracted the interests of scientists involved in the area of cancer therapy. Boswellic acids (BAs), a group of pentacyclic triterpenoids are one such class of molecules which have shown the potential for further development. BAs are mainly isolated from gum resin of *Boswellia serrata* and comprise of four triterpenic acids i.e., β-boswellic acid (BA, **1**), 11-keto-β-boswellic acid (KBA, **2**), acetyl-β-boswellic acid (ABA, **3**) and acetyl-11-keto-β-boswellic acid (AKBA, **4**) [2]. Their bioactivities against inflammation [3,4] arthritis, [5] ulcerative colitis, [6] chronic colitis, [7] asthma [8] and hepatitis [9] are well documented. However, they have gained a great deal of focus in recent past owing to their anti-cancer activity and ability to induce apoptosis [10,11]. BAs have been reported to inhibit growth and induce apoptosis in brain tumours [12] malignant glioma cells, [13] colon cancer cells [14] and leukaemic cells [15]. They have also been found to be more potent inhibitors of topoisomerase I and II in comparison to camptothecin and amsacrine or etopside respectively, using pure topoisomerase assay [16]. The success of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO, I) and its methyl ester (CDDO–Me, II), analogues of oleanolic acid [17] have rejuvenated the interest of medicinal chemists in the triterpenoids. The introduction of 2-cyano-1-en-3-one system into the A ring of their structure such as on glycyrrhetinic acid (III and IV), ursolic acid (V), betulinic acid (VI) and betulin (VII) has generally resulted in a significant enhancement of the apoptotic properties of these molecules, probably due to improved binding at the active sites of the target molecules [18,19]. Notably, the CDDO and its methyl ester (CDDO–Me) are currently in the advance stages of the clinical trials for the cancer therapy [20]. A general look at the structures of the lead triterpenic molecules (Fig. 1) clearly indicated that, besides the presence of 2-cyano-1-en-3-one moieties in A ring, C ring oxygenation in the form of 11/12- keto function was also a key feature in most of these molecules.

2. Results and discussion

Thus inspired by the success of CDDO and our interest in the identification and development of potent anti-cancer leads based on the natural products [21] including boswellic acids [22] we

* Corresponding authors. Tel.: +91(191) 2569011; fax: +91(191) 2569017/2569333.
E-mail addresses: bashah@iiim.ac.in (B.A. Shah), sctaneja@iiim.ac.in (S.C. Taneja).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.01.061

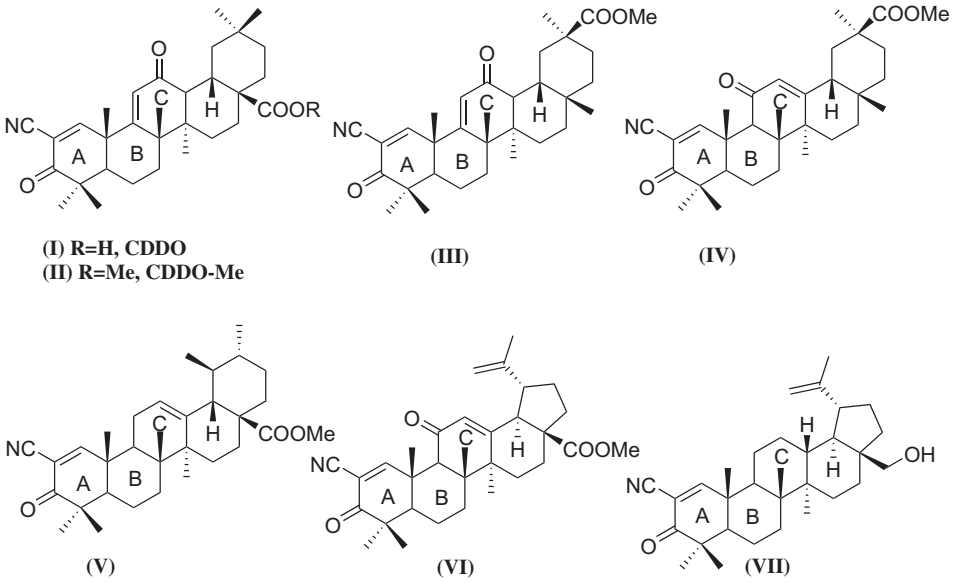
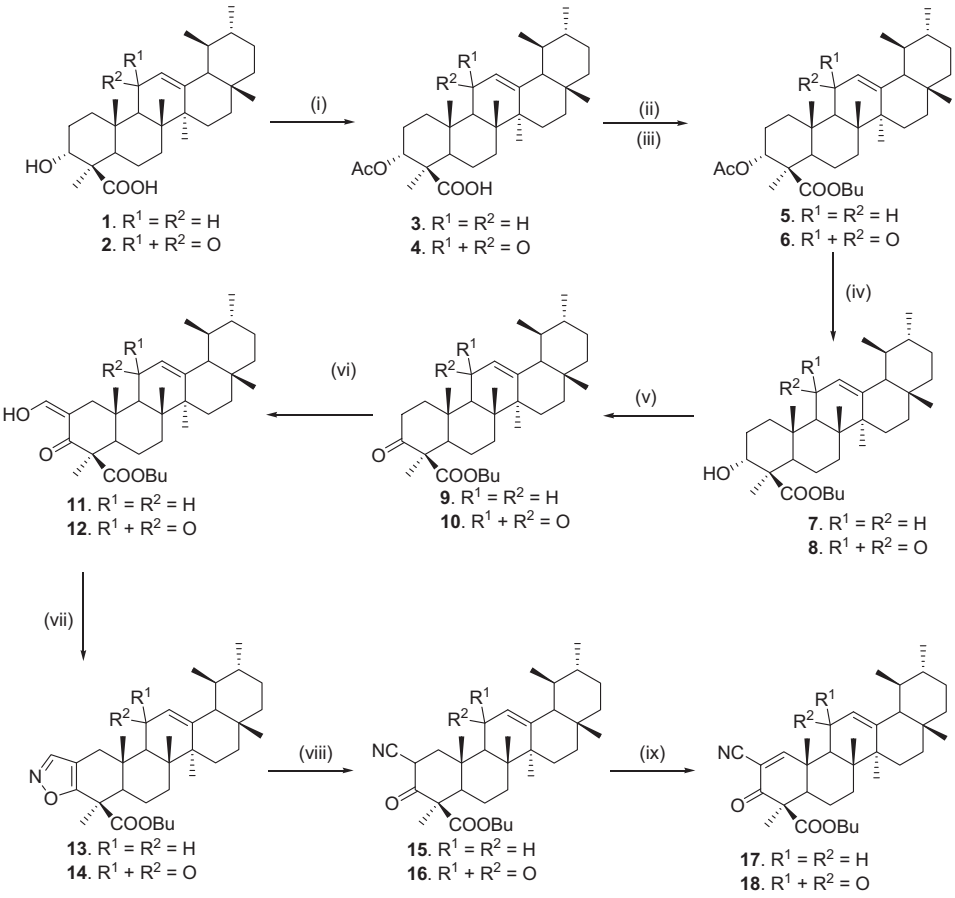
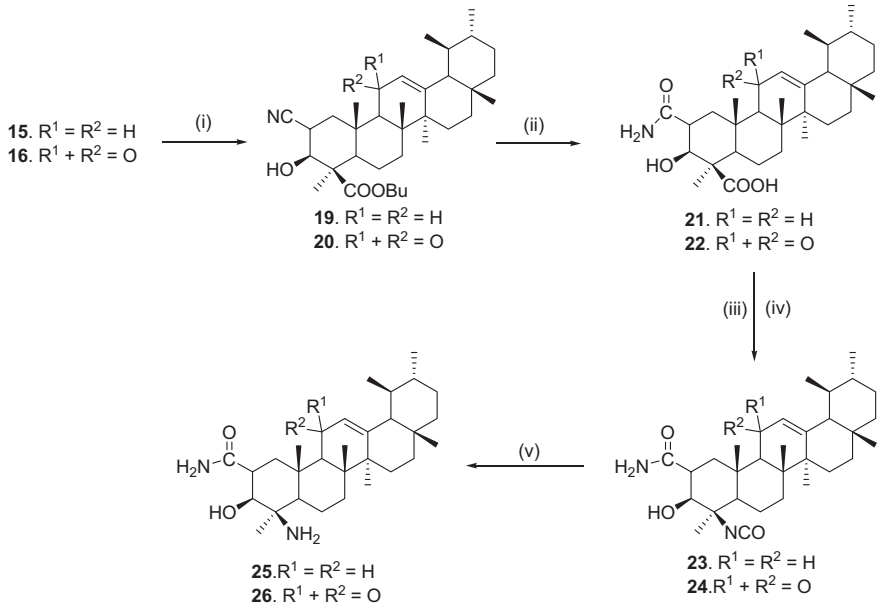


Fig. 1. Cyanoenone derivatives of triterpenes.

envisaged the preparation of 2-cyano-1-en-3-one derivatives of both BA (I) and KBA (II). An earlier report described the preparation of 2-cyano analogues of BA and some other triterpenoids wherein these molecules have displayed significant cytotoxicity against different human cancer cell lines [23]. On the other hand it is generally observed that anti-inflammatory activities of BAs have also been found to be in the order of AKBA as the most active, followed by KBA, ABA and BA in the descending order, thus



Scheme 1. Reagents and conditions: (i) Ac_2O, DMAP, DCM; (ii) $SOCl_2$, Benzene, reflux; (iii) *n*BuOH, 0 °C to rt; (iv) K_2CO_3, MeOH, reflux; (v) PCC, DCM, rt; (vi) HCOOMe, NaH, Benzene, 0 °C to rt; (vii) $NH_2OH.HCl$, EtOH; (viii) NaOMe, MeOH; (ix) DDQ, Benzene.



Scheme 2. Reagents and conditions: (i) $NaBH_4$, MeOH, 0 °C; (ii) KOH, MeOH, reflux; (iii) $SOCl_2$, Benzene, reflux; (iv) NaN_3, acetone, rt; (v) TFA: H_2O (2:1), rt.

supporting the observation that 11-keto function is important for the bioactivity [2]. Besides, in our previous communication, we reported the synthesis of 4-amino analogues of BAs, wherein the 11-keto derivatives (KBA) were found to be far more potent than those of BA [22]. Therefore, the focus of present study has been the lead optimisation through synthesising 2-cyano-1-en-3-one derivatives of both BA and KBA for the comparison of their cytotoxicity and apoptotic potential. Additionally, we also envisaged to introduce nitrogenous group at C-4 position to understand the structure and activity relationship. Thus, a series of semi-synthetic analogues were prepared and screened for their ability to induce cytotoxicity in human cancer cells and also examined for their apoptotic potential.

To begin with, the free carboxylic function in acetyl derivatives of ABA (**3**) and AKBA (**4**) was converted to butyl ester due to its better hydrolysability in a biological system than the methyl ester and also to avoid decarboxylation of β-keto acids during oxidation at C-3. The compounds **3**/**4** were synthesized from BA (**1**) and KBA (**2**) by reacting with acetic anhydride in presence of DMAP as catalyst. The protection of carboxylic group was brought about by the treatment of **3**/**4** with thionyl chloride followed by *in situ* addition of *n*-butanol to produce butyl-3-α-acetoxyurs-12-en-24-oate (**5**) and butyl-3-α-acetoxy-11-oxours-12-en-24-oate (**6**) respectively in 90% yield. The acetyl protection was removed using K_2CO_3 in methanol by refluxing on a water bath, giving butyl-3-α-hydroxyurs-12-en-24-oate (**7**) and butyl-3-α-hydroxy-11-oxours-12-en-24-oate (**8**) respectively in 92% yield. The oxidation of **7**/**8** with PCC in DCM produced butyl-3-oxours-12-en-24-oate (**9**) and butyl-3,11-dioxours-12-en-24-oate (**10**) in 85% yield. The formylation at C-2 of both **9**/**10** was smoothly achieved using NaH and HCOOMe in dry benzene yielding butyl 2-hydroxymethylene-3-oxours-12-en-24-oate (**11**) and butyl 2-hydroxymethylene-3,11-dioxours-12-en-24-oate (**12**) respectively in 90% yields, which were subsequently converted into corresponding isoxazoles i.e., butyl isoxazolo[4,5-*b*]urs-12-en-24-oate (**13**) and butyl 11-oxoisoxazolo [4,5-*b*]urs-12-en-24-oate (**14**) in 95% yields by adding hydroxylamine hydrochloride. The isoxazole moiety was cleaved using NaOMe to effect the formation of butyl 2-cyano-3-oxours-12-en-24-oate (**15**) and butyl 2-cyano-3,11-dioxours-12-en-24-oate (**16**) respectively in 95% yields. Finally the generation of the double bond at C1–C2 was achieved by dehydrogenation with DDQ to obtain butyl 2-cyano-3-oxours-1,12-dien-24-oate (**17**) and butyl 2-cyano-3,11-dioxours-1,12-dien-24-oate (**18**) respectively in 90% yields (Scheme 1).

Table 1
In vitro cytotoxicity of boswellic acid analogues against human cancer cell lines.

Analogues	IC_{50} (μM)	
	HL-60	HeLa
1	24	35
2	32	50
3	10	22
4	11	31
5	28	>50
6	30	40
7	>50	>50
8	34	50
9	>50	>50
10	>50	>50
11	33	44
12	5	>50
13	50	50
14	33	>50
15	14	35
16	11	17
17	**0.42**	**7.9**
18	**0.67**	**3**
19	>50	>50
20	14	30
21	>50	>50
22	>50	>50
23	>50	>50
24	24	45
25	37	>50
26	>50	>50

Note: HL-60 cells 2.0×10^4/200 μl media and HeLa cells 10^4/200 μl media in 96-well culture plate were treated with different concentrations (0.1–50 μM) of each analogue for 48 h, thereafter cultures incubated with MTT for 2 h at 37 °C. Other conditions are described in Materials and methods. IC_{50} value was calculated in terms of 50% of cell viability as compare to untreated control. Data are representative of one of three similar experiments with coefficient of variation of less than 10%.

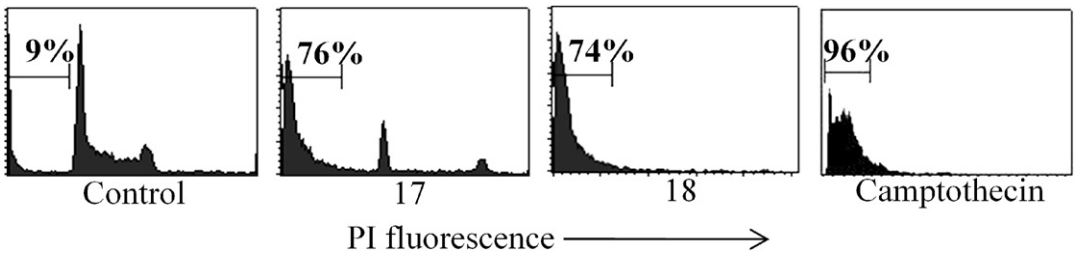


Fig. 2. DNA cell cycle analysis in HL-60 cells, exposed to compound **17** and **18**. HL-60 cells (1×10^6/ml) in culture were treated with compound **17** and **18** at 4 μM for 18 h. Camptothecin, at a concentration of 4 μM for 6 h was used as positive control. Cells were stained with PI to determine DNA fluorescence by flow cytometry as described in Materials and methods. Sub-G1 population indicative of DNA damage was analyzed from the hypo-diploid fraction (<2n DNA) of DNA cell cycle analysis. Data are representative of one of three similar experiments.

We further envisaged establishing the role of carboxylic group in anti-cancer activity of BAs by replacing it with C-4 amino group. Thus 3-keto group in **15**/**16** was reduced using $NaBH_4$ in methanol to produce butyl 2-cyano-3-hydroxyurs-12-en-24-oate (**19**) and butyl 2-cyano-3-hydroxy-11-oxours-12-en-24-oate (**20**) in 90% yield. This was followed by the hydrolysis of **19**/**20** using KOH in methanol to yield 2-carbamoyl-3-hydroxyurs-12-en-24-oic acid (**21**) and 2-carbamoyl-3-hydroxy-11-oxours-12-en-24-oic acid (**22**) respectively in 92% yield. The compounds **21**/**22** were subsequently treated with thionyl chloride followed by *in situ* addition of sodium azide to produce 3-hydroxy-4-isocyanato-urs-12-en-2-carboxamide (**23**) and 3-hydroxy-4-isocyanato-11-oxours-12-en-2-carboxamide (**24**) in 90% yield. The compounds **23**/**24** were converted

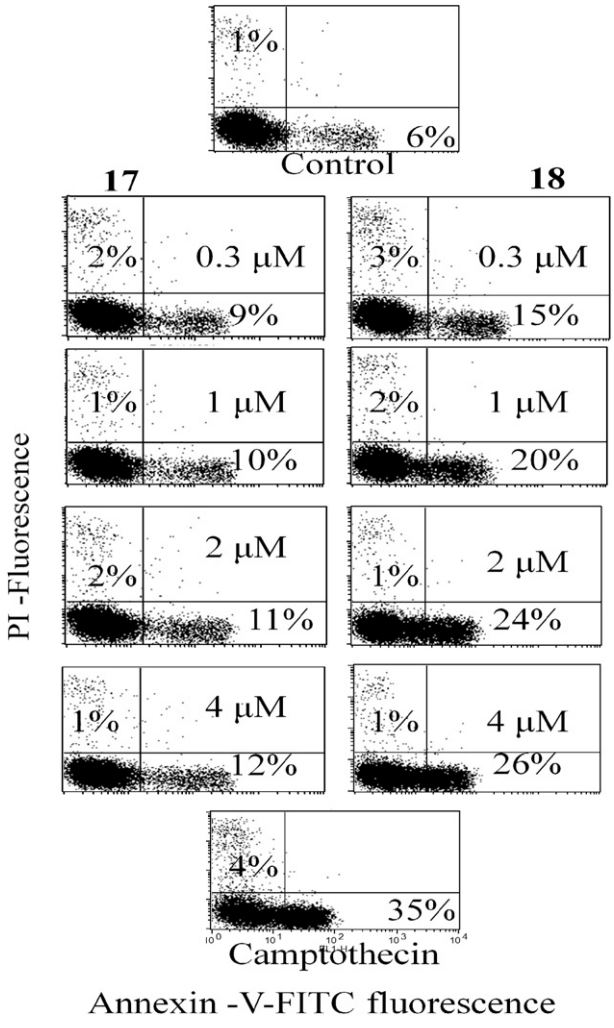


Fig. 3. Flow cytometric analysis of compound **17** and **18** induced apoptosis in HL-60 cells using AnnexinV-FITC/PI. Cells were incubated with indicated concentrations of **17** and **18** for 6 h and stained with AnnexinV-FITC/PI to analyze apoptotic and necrotic cell populations as described in Materials and methods. Camptothecin, at a concentration of 4 μM for 6 h was used as positive control. Data are representative of one of three similar experiments.

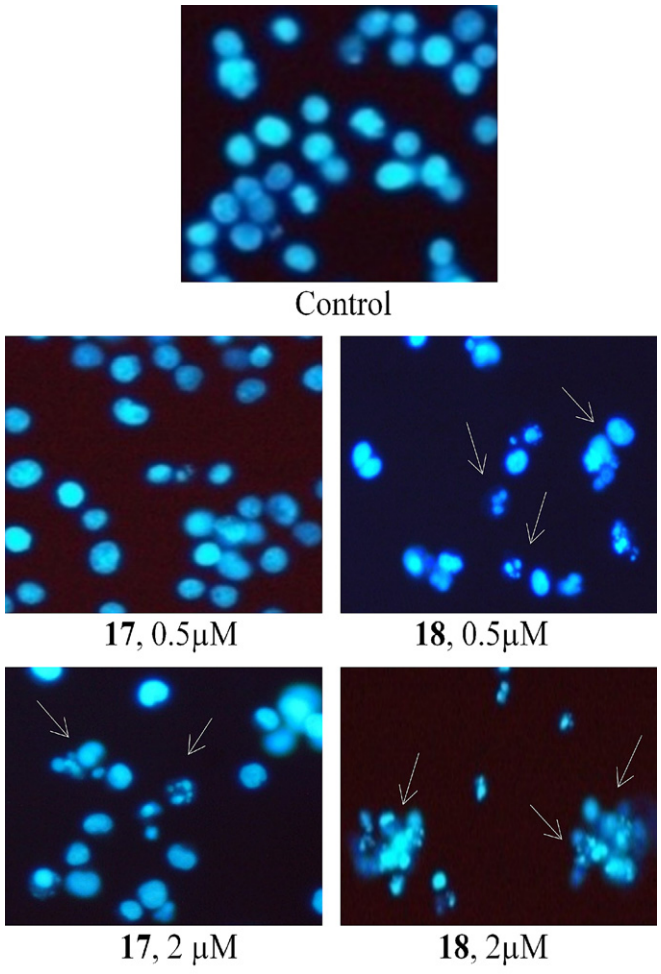


Fig. 4. Effect of compound **17** and **18** on the nuclear morphology of HL-60 cells. HL-60 cells were treated with varied concentrations of **17** and **18** for 9 h and subsequently stained with Hoechst 33258 as described in Materials and methods. Cells were observed under fluorescence microscopy (20×). Both **17** and **18** induced the formation of apoptotic bodies that are indicated by arrows. Data are one of two similar experiments.

into 4-amino-3-hydroxy-urs-12-en-2-carboxamide (**25**) and 4-amino-3-hydroxy-11-oxours-12-en-2-carboxamide (**26**) respectively by treatment with TFA and water in 90% yield (Scheme 2).

The synthesized derivatives were screened for their ability to induce cytotoxicity in two specific human cancer cell lines viz., HL-60 and HeLa cells. The studies indicated the greater sensitivity of HL-60 cells towards cytotoxicity of semi-synthetic analogues than the HeLa cells, as evidenced by the extent of cytotoxicity (IC_{50} values) after 48 h of treatment. The cytotoxicity profiles of all the analogues including intermediates clearly identified the two potential candidates **17** and **18**, displaying IC_{50} values in nanomolar concentrations against HL-60 cells (Table 1). The compounds **17** and **18** exhibited cytotoxicity at lower concentrations in HeLa cells too. DMSO used as delivery vehicle (<0.2% v/v), did not affect the cell growth when treated for the same time period.

Further experiments were carried out to verify whether the cancer cell death induced by the compounds **17** and **18** was apoptotic, as it has now become increasingly evident that although the primary intracellular targets and the pharmacological mechanisms of action of the anti-cancer drugs vary vastly, the drug-induced cell killing is generally mediated by apoptosis [24]. Both **17** and **18** were observed to be potent apoptosis inducers, as evidenced from the measurement of several biological end-points of the apoptosis such as annexinV binding, DNA fragmentation and increase in sub-G0 DNA fraction. Most of the differentiated cells are arrested in the G1 phase but in case of cancer cells, this control is lost and they go on dividing. Thus, the compound **17** and **18** were subjected to hypo-diploid sub-G0 DNA fraction (<2nDNA) analysis as a measure of apoptosis (Fig. 2). HL-60 cells treated with **17** and **18** at 4 μM showed a considerable increase in the hypo-diploid sub-G0 DNA fraction (<2n DNA), i.e. 76% in case of **17** and 96% in case of **18** in just 18 h, indicating DNA damage.

Also, HL-60 cells treated with **17** and **18** at varied concentrations for 6 h underwent an apoptotic cell death as determined through flow cytometry via AnnexinV binding of phosphatidyl serine of exposed cells undergoing apoptosis. The extent of apoptosis vs.

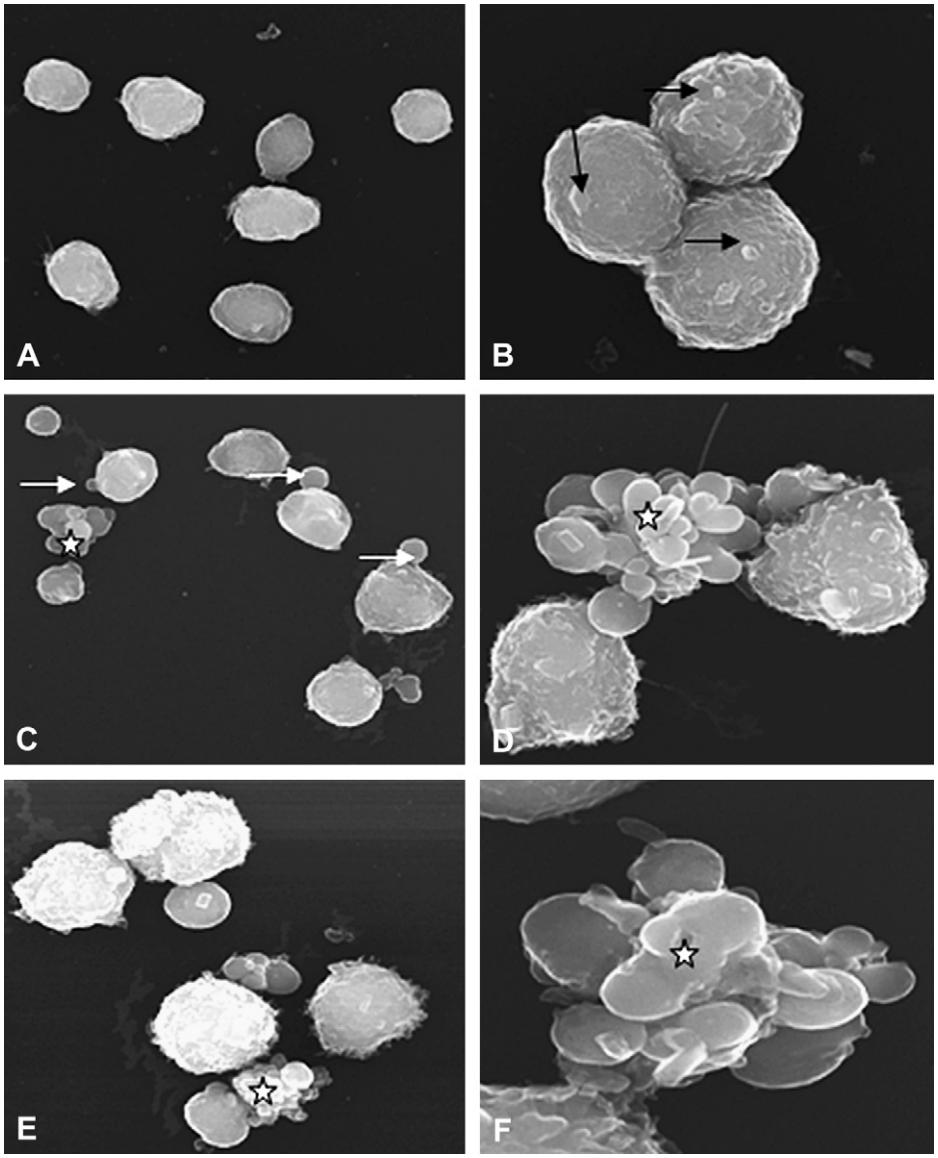


Fig. 5. A–F. Scanning electron microscopy of control (A, B), **17** (C–D) and **18** (E–F) treated HL-60 cells showing surface ultrastructure. The control cells show microvilli on cell surface (B, black arrow), The treatment of **17** (0.5 μM, C; 2 μM, D) and **18** (0.5 μM, E; 2 μM, F) after 9 h causes smoothening of cell surface and blebbing of plasma membrane (C, white arrow) and formation of apoptotic bodies (C–F, asterisk). (Magnification A, C- 2000×; B, D-5000×; E–3000×; F-8000×).

necrosis was analyzed from the quadrant analysis of the cell populations stained with annexinV-FITC/PI. The basal apoptotic population in the untreated culture was 4%, which displayed a concentration dependent increase with both **17** and **18**, the latter eliciting higher apoptotic index than **17** (Fig. 3). Thus, it can easily be inferred from the above experiments that among the two analogues, **18** appeared to have higher apoptotic index in a shorter period of exposure, compared to **17** (Fig. 3). The compound **18** produced a time dependent increase in the apoptotic cell population of about 26%, whereas **17**, besides, having comparatively lesser IC_{50} than **18** showed only 11% of apoptotic cell population at 6 h of incubation. It appeared that **17** require prolonged exposure time to cause apoptotic cell death. Further the PI positive post-apoptotic/necrotic cell population, however, was relatively small, suggesting that **17** and **18** induced cytotoxicity predominantly through apoptotic pathways.

Furthermore, the studies for nuclear morphological changes of HL-60 cells were carried out to gain insight into the morphological alterations caused by these compounds. For this, we employed Hoechst 33258 staining that selectively binds DNA and allows monitoring of nuclear morphological changes under fluorescence microscopy. Both **17** and **18** induced nuclear condensation and blebbing in HL-60 cells after 9 h of treatment where the compound **18** was observed to show increased number of scattered apoptotic bodies formation at even a low concentration of 0.5 μM (Fig. 4). The presence of apoptotic bodies observed in cells treated with **17** and **18** confirmed that they trigger cell demise by apoptosis.

To further corroborate our results, we envisaged to determine apoptotic potential of **17** and **18** through scanning electron microscope (SEM), as the morphological analysis by electron microscopy constitutes a very important and unambiguous method to identify the specific type of cell death and has been regarded as the gold standard for the most precise detection of apoptosis based on the original morphological criteria as described by Wyllie et al. [25]. The use of SEM has also been advocated recently for more accurate assessment of mechanism of cell death in human cancer cells [26]. The cells treated with compound **17** for 9 h at 0.5 μM caused condensation, smoothening of cell surface and blebbing of plasma membrane in most of the cells and apoptotic body formation in a few cells (Fig. 5, C) and at 2 μM the cells shrank due to a loss of cytoplasmic volume and the apoptosis was observed in most of the cells (Fig. 5, D). On the other hand, the cells treated with the compound **18** showed dose dependent surface ultrastructural changes typical of apoptosis such as smoothening of cell surface, decrease in cell size, blebbing of plasma membrane and apoptotic body formation in majority of the cells (Fig. 5, E–F). The results clearly indicated the potential of both **17** and **18** to induce apoptosis and exhibition of anti-proliferative effects on tumour cells. Moreover, **18** appear to be more promising of the two as it was able to trigger significant apoptotic body formation even at 0.5 μM concentration.

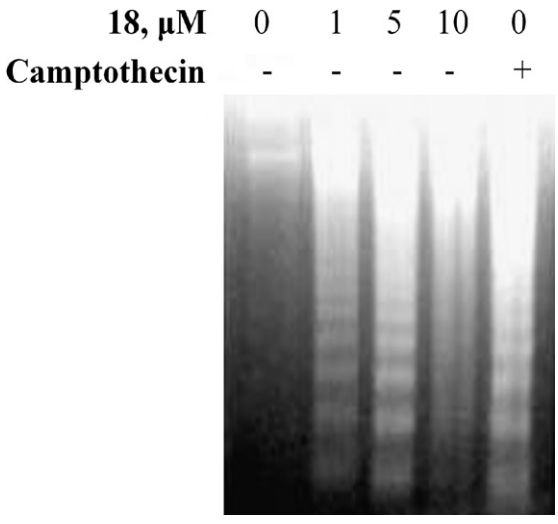


Fig. 6. Compound **18** induced DNA fragmentation in HL-60. Fragmentation of genomic DNA was studied in cells exposed to different concentrations of **18** for 9 h. Camptothecin, at a concentration of 4 μM for 6 h was used as positive control. Genomic DNA was isolated and electrophoresed as described in Materials and methods. Each lane depicts the treatment given, and control and standard used.

Based on the above studies which implied that **18** is a better lead molecule than **17**, we confirmed the apoptotic potential of **18** through induction of DNA fragmentation in HL-60 cells, which is known as the hallmark of apoptosis. Apoptosis typically involves intra-nucleosomal chromatin cleavage by endonucleases in multiples of 180 bp leading to a typical DNA ladder. The compound **18** induced concentration dependent DNA laddering of 180–200 bp in HL-60 cells. The minimal concentration inducing DNA fragmentation was evident at 1 μM. The DNA ladder was diffused interspersed with smear indicative of some post-apoptotic necrosis in HL-60 cells that were treated with higher concentrations of **18**. The positive control used was camptothecin, which at a concentration of 4 μM also exhibited DNA ladder, typical of apoptosis (Fig. 6).

3. Conclusion

In conclusion, the semi-synthetic cyanoenone analogues of BAs were found to have IC_{50} values in nanomolar concentrations in HL-60 and Hela cells. The results also demonstrated the significance of cyanoenone functionality in **17** and **18**, displaying about 50–100 fold higher cytotoxicity than their parent molecules. Moreover the compound **18**, which is derived from KBA has shown an early proapoptotic effect and higher index than **17** as measured by various biological end-points like annexinV binding, DNA laddering, apoptotic bodies formation and an increase in hypo-diploid sub-G0 DNA content during the early 9 h period of study. This thereby substantiates the earlier observations that molecules comprising 11-keto function are generally more potent than those without it [2]. The cyano analogues (**15**, **16** and **19**, **20**) lacking 1-ene functionality were approximately 100 fold less active than **17** and **18**. Thus α, β unsaturation in ring A plays an important role in bioactivity profile of the boswellic acid analogues, implying that ring A is directly involved at the binding site of the receptor. The presence of hydroxylmethyl/formyl group (**11** and **12**), isoxazole moiety (**13** and **14**), 3-keto analogues (**9** and **10**), or esters (**5**–**8**) and other derivatives displayed comparatively lower effect on cytotoxicity. Further investigations for the detailed biological activities including mechanism of action of compound **18** are in progress, which appears to be a potential lead molecule of anti-cancer therapeutic significance.

4. Experimental

4.1. General

MPs were measured in a Buchi-510 apparatus. ^{1}H NMR and ^{13}C NMR spectra in $CDCl_3$ were recorded on Bruker 200, 400 and 500 MHz spectrometers (200, 400 & 500 MHz ^{1}H, 50, 100 & 125 MHz ^{13}C) with TMS as an internal standard. Chemical shifts are expressed in parts per million (δ ppm); *J* values are given in Hertz. Reagents and solvents used were mostly AR grade. Silica gel coated aluminium plates from M/s Merck were used for TLC. MS were recorded on Jeol MSD-300 and Bruker Esquire 3000 GC-Mass spectrometer. Elemental analyses were performed on Elementar

Vario EL-III. Optical rotations were measured on Perkin–Elmer 241 polarimeter at 25 °C using sodium D light.

4.1.1. Butyl 3-α-acetoxy-urs-12-en-24-oate (**5**)

A solution of **3** (1 g, 2.0 mmol) and $SOCl_2$ (0.5 ml, 4.2 mmol) in dry benzene was refluxed for 3 h to prepare corresponding acid chloride. The excess of $SOCl_2$ was distilled out under reduced pressure followed by the addition of *n*-butanol. After the completion of the reaction it was diluted with water; extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant gave **5** (yield 90%) as colourless solid. $[\alpha]_D^{25}$ +15.1 (*c* 0.1, $CHCl_3$), mp 118–120 °C ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.79, 0.80, 0.89, 0.92, 0.94, 1.02, 1.10, 1.16 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 2.07 (s, 3H, $-CO\underline{CH_3}$), 4.09 (m, 2H, $-OCH_2$), 5.15 (brs, 1H, H-12), 5.30 (brs, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.3, 13.6, 16.7, 17.4, 19.3, 19.6, 21.3, 23.2, 23.4, 23.6, 23.7, 27.4, 27.8, 28.7, 30.4, 31.2, 32.3, 33.8, 34.1, 37.2, 38.7, 39.6, 39.7, 39.8, 41.5, 42.8, 46.4, 46.8, 50.6, 59.1, 64.3, 73.5, 123.0, 139.5, 170.2, 175.9. ESI-MS (*m*/*z*): 577 $[M + Na]^+$. Anal. Calc. for $C_{36}H_{58}O_4$: C, 77.93; H, 10.54. Found: C, 77.71; H, 10.72.

4.1.2. Butyl 3-α-acetoxy-11-oxo-urs-12-en-24-oate (**6**)

The compound **6** was prepared from **4** following the same procedure as for **5**. $[\alpha]_D^{25}$ +14.6 (*c* 0.1, $CHCl_3$), mp 126–128 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.82, 0.94, 0.98, 1.06, 1.18, 1.35, 1.39, (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 2.04 (s, 3H, $-CH_3CO\underline{CH_3}$), 4.08 (m, 2H, $-OCH_2$), 5.33 (m, 1H, H-3), 5.55 (m, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 12.6, 12.9, 16.7, 17.6, 18.7, 19.8, 20.4, 20.6, 22.9, 23.0, 23.3, 23.7, 26.5, 26.9, 28.1, 29.7, 29.9, 32.9, 33.2, 34.5, 36.5, 38.6, 40.7, 43.0, 44.3, 45.9, 49.8, 58.3, 59.6, 63.8, 72.6, 129.8, 164.1, 169.5, 174.9, 198.6. ESI-MS (*m*/*z*): 591 $[M + Na]^+$. Anal. Calc. for $C_{36}H_{56}O_5$: C, 76.01; H, 9.92. Found: C, 75.85; H, 10.09.

4.1.3. Butyl 3-α-hydroxy-urs-12-en-24-oate (**7**)

In a solution of **5** (1 g, 1.8 mmol) in methanol: water (80:20) was added K_2CO_3 (2 g) and kept on refluxing till reaction completion. After completion of the reaction, pH was adjusted to 7 using dil. HCl, extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave **7** (yield 92%) as white powder. $[\alpha]_D^{25}$ +41.5 (*c* 0.1, $CHCl_3$), mp 142–146 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.80, 0.87, 0.91, 0.94, 0.98, 1.03, 1.09, 1.28 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 4.05 (m, 3H, H-3 & $-CH_3OCH_2$), 5.14 (brs, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.3, 13.6, 16.7, 17.4, 19.3, 19.7, 21.3, 23.2, 23.3, 23.9, 24.9, 25.4, 27.6, 28.7, 30.4, 31.2, 32.3, 33.7, 33.9, 37.3, 39.5, 39.6, 39.9, 41.5, 42.2, 46.7, 47.5, 48.9, 59.1, 63.9, 70.7, 122.9, 139.4, 177.2. ESI-MS (*m*/*z*): 535 $[M + Na]^+$. Anal. Calc. for $C_{34}H_{56}O_3$: C, 79.63; H, 11.01. Found: C, 79.48; H, 11.13.

4.1.4. Butyl 3-α-hydroxy-11-oxo-urs-12-en-24-oate (**8**)

The compound **8** was prepared from **6** following the same procedure as for **7**. $[\alpha]_D^{25}$ +48.9 (*c* 0.1, $CHCl_3$), mp 168–170 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.81, 0.94, 0.98, 1.05, 1.17, 1.28, 1.31, 1.42 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 3.98 (m, 3H, H-3 & $-CH_3OCH_2$), 5.54 (brs, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.2, 13.6, 17.3, 18.2, 18.8, 19.3, 19.6, 20.5, 21.0, 24.2, 26.2, 27.4, 27.7, 28.7, 30.4, 30.6, 32.1, 33.8, 33.9, 37.3, 39.2, 41.4, 43.7, 45.0, 47.8, 48.7, 58.9, 60.3, 64.0, 70.6, 130.4, 164.8, 174.8, 199.4. ESI-MS (*m*/*z*): 549 $[M + Na]^+$. Anal. Calc. for $C_{34}H_{54}O_4$: C, 77.52; H, 10.33. Found: C, 77.31; H, 10.52.

4.1.5. Butyl 3-oxo-urs-12-en-24-oate (**9**)

Pyridinium chloro chromate (PCC, 2.5 eq.) was added to a solution of compound **7** (1 g, 2 mmol) in DCM and kept on stirring at room temperature till the completion of reaction. The reaction mixture was diluted with water, extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 96:4) as eluant, gave **9** as a white solid in 85% yield. $[\alpha]_D^{25}$ +49.4 (*c* 0.1, $CHCl_3$), mp 104–106 °C ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.74, 0.80, 0.84, 0.86, 0.99, 1.02, 1.04, 1.30 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 3.98 (m, 2H, $-OCH_2$), 5.07 (m, 1H, H-12). ^{13}C NMR (50 MHz, $CDCl_3$): δ 13.8, 13.9, 14.6, 17.2, 17.8, 19.6, 20.9, 21.2, 21.3, 21.7, 23.4, 24.0, 26.9, 28.4, 29.1, 30.7, 31.1, 33.8, 34.1, 36.9, 37.5, 39.9, 40.0, 40.2, 41.8, 42.5, 46.8, 48.9, 58.5, 59.4, 124.4, 140.0, 174.1, 211.3. ESI-MS (*m*/*z*): 533 $[M + Na]^+$. Anal. Calc. for $C_{34}H_{54}O_3$: C, 79.95; H, 10.66. Found: C, 79.72; H, 10.85.

4.1.6. Butyl 3,11-dioxo-urs-12-en-24-oate (**10**)

The compound **10** was prepared from **8** following the same procedure as for **9**. $[\alpha]_D^{25}$ +63.3 (*c* 0.1, $CHCl_3$), mp 120–122 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.78, 0.83, 0.92, 1.22, 1.28, 1.31, 1.35, 1.37 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 4.04 (m, 2H, $-OCH_2$), 5.58 (s, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.3, 13.5, 14.2, 17.3, 18.2, 19.1, 19.6, 20.2, 20.9, 27.2, 27.3, 28.7, 30.2, 31.1, 32.6, 33.8, 36.5, 36.9, 39.1, 40.8, 41.8, 43.6, 44.7, 57.5, 58.2, 58.9, 59.9, 60.0, 64.9, 130.2, 165.2, 173.3, 198.5, 208.1. ESI-MS (*m*/*z*): 547 $[M + Na]^+$. Anal. Calc. for $C_{34}H_{52}O_4$: C, 77.82; H, 9.99. Found: C, 78.04; H, 9.79.

4.1.7. Butyl 2-hydroxymethylen-3-oxo-urs-12-en-24-oate (**11**)

In an ice cooled solution of **9** (900 mg, 1.8 mmol) in dry toluene was added sodium hydride (192 mg, 8 mmol) and methyl formate (0.36 ml, 6 mmol) in small instalments over a period for 30 min. The reaction was kept on stirring overnight. After completion, the reaction mixture was diluted slowly with Et_2O and washed with 1.5 N aq. hydrochloric acid, usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave **11** in 90% yield as white solid. $[\alpha]_D^{25}$ +35.7 (*c* 0.1, $CHCl_3$), mp 112–114 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.79, 0.85, 0.98, 1.02, 1.05, 1.29, 1.42 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 3.95 (m, 2H, $-OCH_2$), 5.12 (brs, 1H, H-12), 8.49 (s, 1H, $=\underline{CH}OH$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.5, 14.4, 15.1, 16.6, 17.4, 19.1, 20.2, 21.3, 23.1, 23.6, 25.2, 26.5, 28.7, 29.6, 30.3, 31.2, 31.9, 32.6, 33.7, 35.8, 39.5, 39.7, 41.4, 42.8, 45.6, 52.2, 53.8, 59.1, 64.8, 106.9, 123.9, 134.3, 139.7, 171.9, 186.7. ESI-MS (*m*/*z*): 561 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{54}O_4$: C, 78.02; H, 10.10. Found: C, 78.30; H, 9.89.

4.1.8. Butyl 2-hydroxymethylene-3,11-dioxo-urs-12-en-24-oate (**12**)

The compound **12** was prepared from **10** following the same procedure as for **11**. $[\alpha]_D^{25}$ +78.5 (*c* 0.1, $CHCl_3$), mp 126–128 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.82, 0.87, 0.90, 0.94, 1.18, 1.25, 1.30, 1.48 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 2.42 (m, 1H, H at C_9), 4.06 (m, 2H, $-OCH_2$), 5.61 (brs, 1H, H-12), 7.26 (s, 1H, $=\underline{CH}OH$). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.6, 14.3, 14.7, 16.9, 17.4, 17.8, 18.2, 19.4, 20.5, 21.1, 25.6, 26.8, 28.9, 29.3, 29.7, 32.3, 36.9, 37.3, 39.3, 40.8, 44.4, 47.3, 51.8, 55.5, 56.4, 59.0, 59.3, 65.2, 106.5, 129.9, 134.6, 166.2, 172.8, 187.8, 198.8. ESI-MS (*m*/*z*): 575 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{52}O_5$: C, 76.05; H, 9.48. Found: C, 76.24; H, 9.28.

4.1.9. Butyl isoxazole[4, 5-b]urs-12-en-24-oate (**13**)

A mixture of **11** (800 mg, 1.5 mmol) and hydroxylamine hydrochloride (210 mg, 3 mmol) in ethanol/water (2:1) (30 ml) was refluxed till reaction completion. After reaction completion, extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave **13** as white solid in 95%

yield. $[\alpha]_D^{25}$ +22.3 (*c* 0.1, $CHCl_3$), mp 113–115 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.81, 0.89, 0.90, 1.04, 1.08, 1.14, 1.26, 1.63 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3CH_3$ & $-CH_3CH_2\underline{CH_3}$), 4.03 (m, 2H, $-CH_3OCH_2$), 5.16 (brs, 1H, H at C_{12}), 8.04 (s, 1H, $-\underline{CH_3}CH{=}N$). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.5, 14.6, 16.6, 17.5, 19.1, 20.0, 21.3, 23.8, 24.5, 24.8, 26.5, 27.3, 28.3, 28.8, 30.5, 31.1, 31.3, 33.3, 34.1, 38.6, 39.9, 41.7, 42.5, 43.8, 45.7, 46.6, 55.8, 59.6, 65.1, 111.0, 124.3, 139.9, 149.8, 167.2, 172.4. ESI-MS (*m*/*z*): 558 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{53}NO_3$: C, 78.46; H, 9.97; N, 2.61. Found: C, 78.23; H, 10.13; N, 2.72.

4.1.10. Butyl 11-oxoisoxazole[4,5-b]urs-12-en-24-oate (**14**)

The compound **14** was prepared from **12** following the same procedure as for **13**. $[\alpha]_D^{25}$ +44.3 (*c* 0.1, $CHCl_3$), mp 109–111 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.80, 0.85, 0.92, 1.07, 1.17, 1.23, 1.28, 1.61 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 3.97 (m, 2H, $-OCH_2$), 5.56 (brs, 1H, H-12), 8.01 (s, 1H, -CH=N). ^{13}C NMR (50 MHz, $CDCl_3$): δ 13.9, 15.4, 17.8, 18.2, 19.4, 20.8, 21.5, 23.1, 24.8, 27.7, 27.9, 29.3, 30.1, 30.6, 31.3, 32.3, 34.4, 36.7, 38.2, 39.7, 39.8, 41.3, 44.2, 45.2, 55.4, 59.5, 60.2, 65.5, 111.6, 130.8, 150.4, 166.3, 166.7, 172.9, 199.1. ESI-MS (*m*/*z*): 572 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{51}NO_4$: C, 76.46; H, 9.35; N, 2.55. Found: C, 76.21; H, 9.53; N, 2.64.

4.1.11. Butyl 2-cyano-3-oxo-urs-12-en-24-oate (**15**)

Sodium methoxide (2 g, 37 mmol) was added to a solution of **13** (700 mg, 1.3 mmol) in MeOH and Et_2O at 0 °C. The mixture was stirred for 1 h and slowly brought it to room temperature and kept it on stirring for 4–5 h. Product formed was extracted with DCM and was purified by column chromatography. After completion, treatment with 1.5 N aq. hydrochloric acid, extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave **15** (yield 95%) as white solid. $[\alpha]_D^{25}$ +12.3 (*c* 0.1, $CHCl_3$), mp 116–118 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.80, 0.87, 0.91, 0.94, 1.05, 1.07, 1.09, 1.43 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 4.11 (m, 2H, $-OCH_2$), 5.14 (brs, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.6, 13.7, 16.8, 17.5, 20.5, 20.7, 21.4, 23.2, 23.6, 23.7, 24.8, 28.0, 28.8, 30.3, 31.2, 32.3, 33.8, 34.2, 34.6, 36.8, 37.6, 39.6, 39.7, 41.5, 44.2, 46.4, 57.9, 58.1, 59.1, 65.7, 116.9, 123.4, 140.2, 145.6, 173.0. ESI-MS (*m*/*z*): 549 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{53}NO_3$: C, 78.46; H, 9.97; N, 2.61. Found: C, 78.26; H, 10.11; N, 2.69.

4.1.12. Butyl 2-cyano-3,11-dioxo-urs-12-en-24-oate (**16**)

The compound **16** was prepared from **14** following the same procedure as for **15**. $[\alpha]_D^{25}$ +38.7 (*c* 0.1, $CHCl_3$), mp 122–124 °C ^{1}H NMR (200 MHz, $CDCl_3$) : δ 0.81, 0.94, 1.21, 1.25, 1.28, 1.31, 1.54, 2.04 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 4.10 (m, 2H, $-OCH_2$), 5.57 (brs, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.4, 13.5, 17.3, 18.2, 19.1, 19.4, 20.3, 20.7, 21.0, 22.6, 27.2, 28.3, 28.8, 29.6, 30.2, 31.2, 31.4, 33.9, 37.1, 39.0, 39.2, 40.9, 43.8, 44.7, 45.1, 57.8, 58.9, 59.3, 65.7, 116.5, 129.9, 166.2, 172.4, 173.9, 198.1. ESI-MS (*m*/*z*): 572 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{51}NO_4$: C, 76.46; H, 9.35; N, 2.55. Found: C, 76.25; H, 9.50; N, 2.62.

4.1.13. Butyl 2-cyano-3-oxo-urs-1,12-dien-24-oate (**17**)

A mixture of **15** (500 mg, 0.93 mmol) and DDQ (300 mg, 1.3 mmol) in dry toluene was heated under reflux for 1 h. Reaction mixture was then subjected to filtration. The filtrate obtained was evaporated under vacuum and was subjected to chromatography on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave **17** in 90% yields as white solid. $[\alpha]_D^{25}$ +34.7 (*c* 0.1, $CHCl_3$), mp 135–138 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.80, 0.83, 0.88, 1.07, 1.11, 1.16, 1.18, 1.52 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 3.90 (m, 2H, $-OCH_2$), 5.17 (brs, 1H, H-12), 7.66 (s, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.7, 14.3, 17.6, 19.2, 19.4, 20.9, 21.4, 23.2, 23.8, 26.5, 27.9, 28.5, 30.2, 31.2, 32.8, 33.4, 33.9, 34.8, 37.1, 39.6, 41.5, 42.7, 46.7, 47.4, 54.4, 54.8, 59.2, 65.7, 113.8, 120.1, 122.7, 140.8, 167.9, 172.3, 190.5. ESI-MS (*m*/*z*): 556 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{51}NO_3$: C, 78.75; H, 9.63; N, 2.62. Found: C, 78.47; H, 9.82; N, 2.72.

4.1.14. Butyl 2-cyano-3,11-dioxo-urs-1,12-dien-24-oate (**18**)

The compound **18** was prepared from **16** following the same procedure as for **17**. $[\alpha]_D^{25}$ +58.0 (*c* 0.1, $CHCl_3$), mp 156–158 °C ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.79, 0.91, 1.23, 1.28, 1.30, 1.41, 1.52, 1.58 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 4.03 (m, 2H, $-OCH_2$), 5.67 (brs, 1H, H-12), 8.54 (s, 1H, H-1). ^{13}C NMR (125 MHz, $CDCl_3$): δ 13.6, 15.4, 17.4, 18.6, 18.8, 19.1, 20.4, 20.8, 21.1, 27.2, 28.9, 29.4, 29.7, 30.8, 31.8, 32.1, 33.8, 39.2, 39.9, 40.8, 44.1, 45.4, 53.6, 54.0, 54.7, 59.1, 65.8, 113.3, 114.6, 129.6, 167.6, 170.3, 171.9, 190.1, 197.5. ESI-MS (*m*/*z*): 570 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{49}O_4$: C, 76.74; H, 9.02; N, 2.56. Found: C, 77.02; H, 9.21; N, 2.67.

4.1.15. Butyl 2-cyano-3-hydroxy-urs-12-en-24-oate (**19**)

A solution of **17** (500 mg) in MeOH (20 ml), sodium borohydride (350 mg) was added at 0 °C. After completion the reaction was diluted with water, extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave compound **19** in 90% yield as white powder. $[\alpha]_D^{25}$ +37.0 (*c* 0.1, $CHCl_3$), mp 146–148 °C ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.79, 0.83, 0.87, 0.94, 0.97, 1.01, 1.07, 1.12 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 3.23 (brs, 1H, H-2), 3.67 (brs, 1H, H-3), 4.08 (m, 2H, $-OCH_2$), 5.14 (brs, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.3, 13.6, 16.6, 16.7, 17.5, 21.4, 22.3, 22.5, 23.4, 23.6, 25.8, 26.3, 26.9, 28.4, 28.7, 31.1, 32.6, 33.8, 34.7, 37.4, 39.6, 41.4, 41.8, 42.3, 46.7, 47.2, 53.5, 56.1, 59.1, 63.8, 71.2, 121.1, 121.5, 139.9, 179.8. ESI-MS (*m*/*z*): 560 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{55}NO_3$: C, 78.16; H, 10.31; N, 2.60. Found: C, 77.89; H, 10.54; N, 2.45.

4.1.16. Butyl 2-cyano-3-hydroxy-11-oxo-urs-12-en-24-oate (**20**)

The compound **20** was prepared from **18** following the same procedure as for **19**. $[\alpha]_D^{25}$ +48.0 (*c* 0.1, $CHCl_3$), mp 157–159 °C ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.78, 0.83, 0.92, 0.95, 1.20, 1.28, 1.39, 1.49 (24H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$ & $-CH_2\underline{CH_3}$), 3.30 (1H, H-2), 3.75 (1H, H-3), 4.10 (m, 2H, $-OCH_2$), 5.58 (brs, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.5, 16.9, 18.6, 19.4, 20.6, 21.1, 22.8, 25.2, 25.5, 27.5, 27.8, 28.9, 31.1, 31.8, 33.1, 34.1, 37.3, 37.8, 39.5, 39.6, 40.9, 41.1, 44.1, 45.0, 47.9, 56.8, 59.3, 60.8, 63.9, 75.8, 120.6, 130.6, 164.9, 176.1, 197.7. ESI-MS (*m*/*z*): 574 $[M + Na]^+$. Anal. Calc. for $C_{35}H_{53}NO_4$: C, 76.18; H, 9.68; N, 2.54. Found: C, 76.28; H, 9.91; N, 2.72.

4.1.17. 2-Carbamoyl-3-hydroxy-urs-12-en-24-oic acid (**21**)

Potassium hydroxide was added (170 mg) to a solution of **19** (400 mg) in methanol: water (80:20) and kept it on refluxing. After completion of the reaction, pH was adjusted to 7 using dil. HCl, extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave **21** (yield 92%). $[\alpha]_D^{25}$ +40.7 (*c* 0.1, $CHCl_3$), mp 174–176 °C ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.78, 0.87, 0.96, 0.98, 1.02, 1.06, 1.12 (21H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$), 3.10 (brs, 1H, H-2), 4.06 (m, 1H, H-3), 5.11 (m, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 11.3, 13.6, 14.2, 16.7, 17.4, 20.1, 21.3, 22.3, 25.7, 26.1, 26.9, 28.1, 28.8, 32.5, 33.7, 34.8, 37.3, 39.7, 41.5, 42.8, 46.7, 47.5, 48.8, 56.2, 59.1, 63.5, 69.5, 121.5, 139.6, 177.9, 178.5. ESI-MS (*m*/*z*): 522 $[M + Na]^+$. Anal. Calc. for $C_{31}H_{49}NO_4$: C, 74.51; H, 9.88; N, 2.80. Found: C, 74.28; H, 10.13; N, 2.97.

4.1.18. 2-Carbamoyl-3-hydroxy-11-oxours-12-en-24-oic acid (**22**)

The compound **22** was prepared from **20** following the same procedure as for **21**. $[\alpha]_D^{25}$ +39.5 (*c* 0.1, $CHCl_3$), mp 190–192 °C ^{1}H

NMR (500 MHz, $CDCl_3$): δ 0.81, 0.93, 0.94, 1.09, 1.16, 1.29, 1.45 (21H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$), 3.21 (brs, 1H, H-2), 4.06 (m, 1H, H-3), 5.56 (brs, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 17.4, 18.2, 20.4, 21.1, 22.3, 22.4, 23.7, 25.3, 27.2, 27.5, 28.8, 30.9, 32.1, 33.9, 37.1, 39.3, 40.9, 41.9, 43.8, 44.8, 48.7, 56.1, 59.1, 60.0, 63.6, 70.0, 130.4, 165.0, 177.5, 178.3, 198.0. ESI-MS (*m*/*z*): 536 $[M + Na]^+$. Anal. Calc. for $C_{31}H_{47}NO_5$: C, 72.48; H, 9.22; N, 2.73. Found: C, 72.70; H, 8.96; N, 2.97.

4.1.19. 3-Hydroxy-4-isocyanato-urs-12-en-2-carboxamide (**23**)

A solution of **21** (250 mg, 2.0 mmol) and $SOCl_2$ (0.2 ml, 4.2 mmol) in dry benzene was refluxed for 3 h to prepare corresponding acid chloride. The excess of $SOCl_2$ was distilled under reduced pressure followed by addition of acetone and sodium azide. After completion of the reaction it was diluted with water, extraction with DCM (3 × 100 ml), usual workup and chromatography of the crude product on a silica gel column using hexane: ethyl acetate (99:1 to 90:10) as eluant, gave **5** (yield 90%) as colourless solid. $[\alpha]_D^{25}$ +35.1 (*c* 0.1, $CHCl_3$), mp 114–116 °C ^1H NMR (500 MHz, $CDCl_3$): δ 0.78, 0.83, 0.87, 0.93, 0.99, 1.04, 1.07, (21H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$), 3.19 (brs, 1H, H-2), 4.10 (brs, 1H, H-3), 5.11 (m, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.7, 16.9, 17.0, 22.3, 22.4, 23.2, 23.5, 23.6, 23.8, 25.2, 28.7, 31.1, 31.2, 32.5, 33.7, 37.1, 39.5, 39.6, 40.0, 42.2, 51.8, 51.9, 57.6, 58.9, 63.4, 69.4, 89.3, 121.1, 123.8, 140.0, 173.1. ESI-MS (*m*/*z*): 519 $[M + Na]^+$. Anal. Calc. for $C_{31}H_{48}N_2O_3$: C, 74.96; H, 9.74; N, 5.64. Found: C, 74.71; H, 9.97; N, 5.45.

4.1.20. 3-Hydroxy-4-isocyanato-11-oxours-12-en-2-carboxamide (**24**)

The compound **24** was prepared from **22** following the same procedure as for **23**. $[\alpha]_D^{25}$ +34.6 (*c* 0.1, $CHCl_3$), mp 127–129 °C ^1H NMR (200 MHz, $CDCl_3$): δ 0.81, 0.92, 1.14, 1.18, 1.25, 1.31, 1.40 (21H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$), 3.19 (s, 1H, H-2), 4.11 (brs, 1H, H-3), 5.56 (m, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.8, 17.5, 18.8, 20.5, 21.1, 22.4, 23.7, 25.2, 27.2, 27.4, 28.8, 29.7, 30.8, 33.9, 39.2, 39.3, 40.8, 42.0, 42.8, 43.9, 45.3, 51.7, 59.0, 60.2, 63.6, 88.9, 124.1, 130.1, 165.8, 172.8, 198.6. ESI-MS (*m*/*z*): 533 $[M + Na]^+$. Anal. Calc. for $C_{31}H_{46}N_2O_4$: C, 72.91; H, 9.08; N, 5.49. Found: C, 72.77; H, 9.31; N, 5.35.

4.1.21. 4-Amino-3-hydroxy-urs-12-en-2-carboxamide (**25**)

TFA: water (1:1; 5 ml) solution of **23** (200 mg, 1.5 mmol) was allowed to stir at rt for 3 h. The solvents were removed and the crude product purification by silica gel column chromatography gave compound **25** colourless solid in 90% yield. $[\alpha]_D^{25}$ +43.2 (*c* 0.1, $CHCl_3$), mp 100–102 °C ^1H NMR (500 MHz, $CDCl_3$): δ 0.78, 0.84, 0.87, 0.95, 0.99, 1.04, 1.29, (21H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$), 3.48 (brs, 1H, H-2), 4.06 (m, 1H, H-3), 5.14 (m, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 15.3, 16.7, 17.2, 18.9, 20.6, 21.3, 21.8, 22.4, 22.9, 23.4, 23.8, 24.6, 25.2, 28.1, 28.8, 30.3, 31.2, 32.5, 33.7, 37.1, 38.5, 39.6, 40.0, 42.2, 51.9, 58.9, 86.3, 122.8, 145.6, 174.8. ESI-MS (*m*/*z*): 493 $[M + Na]^+$. Anal. Calc. for $C_{30}H_{50}N_2O_2$: C, 76.55; H, 10.71; N, 5.95. Found: C, 76.86; H, 10.54; N, 6.08.

4.1.22. 4-Amino-3-hydroxy-11-oxours-12-en-2-carboxamide (**26**)

The compound **26** was prepared from **24** following the same procedure as for **25**. $[\alpha]_D^{25}$ +46.8 (*c* 0.1, $CHCl_3$), mp 108–110 °C ^1H NMR (500 MHz, $CDCl_3$): δ 0.80, 0.94, 1.13, 1.19, 1.27, 1.34, 1.42 (21H, 23, 25, 26, 27, 28, 29 & 30 $-CH_3$), 3.48 (brs, 1H, H-2), 4.10 (m, 1H, H-3), 5.58 (m, 1H, H-12). ^{13}C NMR (125 MHz, $CDCl_3$): δ 14.9, 17.2, 18.5, 20.1, 21.6, 22.5, 23.8, 25.1, 26.5, 27.4, 28.2, 29.5, 30.2, 33.4, 36.2, 39.5, 40.6, 42.0, 42.8, 43.8, 45.4, 50.9, 59.2, 60.6, 63.6, 88.9, 130.1, 166.4, 173.9, 199.8. ESI-MS (*m*/*z*): 507 $[M + Na]^+$. Anal. Calc. for $C_{30}H_{48}N_2O_3$: C, 74.34; H, 9.98; N, 5.78. Found: C, 74.13; H, 10.21; N, 5.83.

4.2. Methods for biological screening

4.2.1. Chemicals and Antibodies

RPMI-1640 medium, propidium iodide (PI), DNase-free RNase, proteinaseK, 3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazolium bromide (MTT), foetal bovine serum(#F0926, lot No.093K0383) and Hoechst 33258 were purchased from Sigma chemical Co., USA. AnnexinV-FITC apoptosis detection kit was from Santa Cruz Biotechnology, USA. glutaraldehyde and osmium tetroxide were procured from Merck Darmstadt, Germany.

4.2.2. Cell culture, growth conditions and treatment

Human promyelocytic leukaemia cell line, HL-60, was procured from National Centre for Cell Sciences (NCCS), Pune, India and Human cervix carcinoma cell line, Hela, procured from National Cancer Institute, Frederick, U.S.A. Cells were grown in RPMI-1640/MEM medium containing 10% FCS, 1% antibiotic and antimycotic solution, pyruvic acid (0.11 mg/ml), and 0.37% NaHCO3 at 37 °C. Cells were grown in CO_2 incubator (Thermocon Electron Corporation, USA) at 37 °C with 98% humidity and 5% CO_2 gas environment. Cells were treated with analogues dissolved in DMSO, while the untreated control cultures received only the vehicle (DMSO, <0.2%).

4.2.3. Assay of cell proliferation by MTT

HL-60 cells, 2×10^4/200 μl and Hela cells, 1×10^4/200 μl were grown in 96-well plates and were exposed to indicated concentrations of boswellic analogues for 48 h. Then 20 μl of MTT solution (2.5 mg/ml) was added to each well 3 h before the completion of incubation time of 48 h at 37 °C. The plates were centrifuged and the supernatants discarded, while the MTT-formazon crystals were dissolved in 150 μl DMSO. The OD was measured at 570 nm with reference wavelength of 620 nm [27]. Cell growth, as percent viability, was calculated by comparing the absorbance of treated verses untreated cells.

4.2.4. Cell cycle analysis by flow cytometry

HL-60 cells (1×10^6/ml) were treated with **17** and **18**, at a concentration of 4 μM each and incubated for 18 h to investigate the hypo-diploid sub-G0/G1 fraction as a measure of apoptosis. Camptothecin, at a concentration of 4 μM for 6 h, was used as positive control. The cells were harvested at 160 × g for 5 min and the cell pellets washed with PBS, fixed in cold 70% alcohol overnight at −20 °C, digested with DNase-free RNase (400 μg/ml) and stained with propidium iodide as described earlier [27]. Cells were analyzed immediately on an LSR flow cytometer (Becton Dickinson, USA). The fluorescence intensity of sub-G0/G1 cell fraction represents the apoptotic cell population [28].

4.2.5. Hoechst 33258 staining of cells for nuclear morphology

HL-60 cells (2×10^6/3 ml), treated with **17** and **18** for 9 h, were washed twice with PBS, fixed and stained with Hoechst 33258, as described earlier [21b]. The slides were observed for any nuclear morphological alterations and apoptotic bodies under inverted fluorescence microscope (Olympus IX70).

4.2.6. Flow cytometric analysis of apoptosis and necrosis

HL-60 cells (1×10^6/ml) were treated with **17** and **18** for 8 h and 6 h, respectively, at varied concentrations to understand the extent of apoptosis. Camptothecin, at a concentration of 4 μM for 6 h, was used as positive control. Cells were stained with annexinV-FITC antibody and propidium iodide as per instructions of the manufacturer (BD Biosciences). Cells were scanned in FL-1 (FITC) versus FL-2 (PI) channels on BD-LSR flow cytometer, using quadrant statistics for apoptotic and necrotic cell populations [21b].

4.2.7. DNA agarose gel electrophoresis

DNA fragmentation, typical of apoptosis, is the hallmark of cells undergoing apoptotic process. 2×10^6 HL-60 cells were treated with indicated concentrations of **18** for 9 h. The negative control was without treatment whereas the positive control was treated with 4 μM of camptothecin. After incubation, the cells were washed in PBS containing 10 mM EDTA. The cell pellets were lysed, extracted of genomic DNA and precipitated to the DNA pellet. The DNA pellet was washed in 80% alcohol, dried, dissolved in 50 μl TE buffer and electrophoresed in 1.6% agarose gel at 50 V, stained with ethidium bromide and visualized in Bio-Rad gel documentation system as described earlier [21b].

4.2.8. SEM studies

HL-60 cells were seeded in 6-well tissue culture plates at the density of 2×10^5/ml in complete medium supplemented with 10% FCS in the presence and absence (as controls) of **17** and **18** respectively at 0.5 and 2 μM concentrations. The stock solution was prepared in DMSO and added to the medium to achieve the desired final concentration. Control samples were treated with DMSO vehicle alone. After the incubation of 9 h at 37 °C, the HL-60 cells were processed for SEM studies [26]. Briefly the cells on cover slip were fixed immediately with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 1 h, post-fixed with 1% OsO_4 for 1 h in the same buffer, dehydrated in acetone and dried in a critical point drier using CO_2 (Blazer's Union) and coated with gold using a Sputter coater (Polaron). The specimens were examined with a JEOL-100CXII electron microscope with ASID at 40 kV.

Acknowledgement

One of the authors (RK) thanks CSIR, New Delhi for the grant of Junior Research Fellowship. Technical assistance from Mr. Samar S. Andotra is gratefully acknowledged.

References

[1] K.M. Debatin, Activation of apoptosis pathways by anticancer treatment, Toxicol. Lett. 112 (2000) 41–48.
[2] B.A. Shah, G.N. Qazi, S.C. Taneja, Boswellic acids: a group of medicinally important compounds, Nat. Prod. Rep. 26 (2009) 72–89 (references cited therein).
[3] (a) S. Singh, A. Khajuria, S.C. Taneja, R.K. Khajuria, J. Singh, G.N. Qazi, Boswellic acids and glucosamine show synergistic effect in preclinical anti-inflammatory study in rats, Bioorg. Med. Chem. Lett. 17 (2007) 3706–3711;
(b) S. Singh, A. Khajuria, S.C. Taneja, R.K. Johri, J. Singh, G.N. Qazi, Boswellic acids: a leukotriene inhibitor also effective through topical application in inflammatory disorders, Phytomedicine 15 (2008) 400–407.
[4] H.P. Ammon, Boswellic acids in chronic inflammatory diseases, Planta Med. 72 (2006) 1100–1116.
[5] D. Khanna, G. Sethi, K.S. Ahn, M.K. Pandey, A.B. Kunnumakkara, B. Sung, A. Aggarwal, B.B. Aggarwal, Natural products as a gold mine for arthritis treatment, Curr. Opin. Pharmacol. 7 (2007) 344–351.
[6] (a) I. Gupta, A. Parihar, P. Malhotra, G.B. Singh, R. Ludtke, H. Safayhi, H.P. Ammon, Effects of *Boswellia serrata* gum resin in patients with ulcerative colitis, Eur. J. Med. Res. 2 (1997) 37–43;
(b) S. Singh, A. Khajuria, S.C. Taneja, R.K. Khajuria, J. Singh, R.K. Johri, G.N. Qazi, The gastric ulcer protective effect of boswellic acids, a leukotriene inhibitor from *Boswellia serrata*, in rats, Phytomedicine 15 (2008) 408–415.
[7] I. Gupta, A. Parihar, P. Malhotra, S. Gupta, R. Ludtke, H. Safayhi, H.P. Ammon, Effects of gum resin of *Boswellia serrata* in patients with chronic colitis, Planta Med. 67 (2001) 391–395.
[8] A. Gupta, V. Gupta, A. Parihar, S. Gupta, R. Ludtke, H. Safayhi, H.P. Ammon, Effects of *Boswellia serrata* gum resin in patients with bronchial asthma: results of a double-blind, placebo-controlled, 6-week clinical study, Eur. J. Med. Res. 3 (1998) 511–514.
[9] H. Safayhi, T. Mack, H.P. Ammon, Protection by boswellic acids against galactosamine/ endotoxin-induced hepatitis in mice, Biochem. Pharmacol. 41 (1991) 1536–1537.
[10] R. Han, Highlight on the studies of anticancer drugs derived from plants in China, Stem Cells 12 (1994) 53–63.
[11] S. Bhushan, A. Kumar, F. Malik, S.S. Andotra, V.K. Sethi, I.P. Kour, S.C. Taneja, G.N. Qazi, J. Singh, A triterpenediol from *Boswellia serrata* induces apoptosis through both the intrinsic and extrinsic apoptotic pathways in human leukemia HL-60 cells, Apoptosis 12 (2007) 1911–1926.
[12] (a) G. Jannsen, U. Bode, H. Breu, B. Dohm, V. Engelbrecht, U. Gobel, Boswellic acids in the palliative therapy of children with progressive or relapsed brain tumors, Klin. Padiatr. 212 (2000) 189–195;
(b) M. Winkling, S. Sarikaya, A. Rahmanian, A. Jodicke, D.K. Boker, Boswellic acids inhibit glioma growth: a new treatment option? J. Neurooncol. 46 (2000) 97–103.
[13] T. Glaser, S. Winter, P. Groscurth, H. Safayhi, E.R. Sailer, H.P. Ammon, Boswellic acids and malignant glioma: induction of apoptosis but no modulation of drug sensitivity, Br. J. Cancer 80 (1999) 756–765.
[14] J.J. Liu, A. Nilsson, S. Oredsson, V. Badmaev, W.Z. Zhao, R.D. Duan, Boswellic acids trigger apoptosis via a pathway dependent on caspase-8 activation but independent on Fas/Fas ligand interaction in colon cancer HT-29 cells, Carcinogenesis 23 (2002) 2087–2093.
[15] (a) R.F. Hoernlein, T. Orlikowsky, C. Zehrer, D. Niethammer, E.R. Sailer, T. Simmet, G.E. Dannecker, H.P. Ammon, Acetyl-11-keto-beta-boswellic acid induces apoptosis in HL-60 and CCRF-CEM cells and inhibits topoisomerase I, J. Pharmacol. Exp. Ther. 288 (1999) 613–619;
(b) Y. Jing, S. Nakajo, L. Xia, K. Nakaya, Q. Fang, S. Waxman, R. Han, Boswellic acid acetate induces differentiation and apoptosis in leukemia cell lines, Leuk. Res. 23 (1999) 43–50.
[16] T. Syrovets, B. Buchele, E. Gedig, J.R. Slupsky, T. Simmet, Acetyl-boswellic acids are novel catalytic inhibitors of human topoisomerases I and IIα, Mol. Pharmacol. 58 (2000) 71–81.
[17] (a) T. Honda, B.V. Rounds, G.W. Gribble, N. Suh, Y. Wang, M.B. Sporn, Design and synthesis of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages, Bioorg. Med. Chem. Lett. 8 (1998) 2711–2714;
(b) T. Honda, G.W. Gribble, N. Suh, H.J. Finlay, B.V. Rounds, L. Bore, F.G. Favarolo Jr., Y. Wang, M.B. Sporn, Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages, J. Med. Chem. 43 (2000) 1866–1877.
[18] (a) G. Chadalapaka, I. Jutooru, A. McAlees, T. Stefanac, S. Safe, Structure-dependent inhibition of bladder and pancreatic cancer cell growth by 2-substituted glycyrrhetinic and ursolic acid derivatives, Bioorg. Med. Chem. Lett. 18 (2008) 2633–2639;
(b) S. Chintharlapalli, S. Papineni, I. Jutooru, A. McAlees, S. Safe, Structure-dependent activity of glycyrrhetinic acid derivatives as peroxisome proliferator-activated receptor {gamma} agonists in colon cancer cells, Mol. Cancer Ther. 6 (2007) 1588–1598.
[19] (a) Y.-J. You, Y. Kim, N.-H. Nam, B.-Z. Ahn, Synthesis and cytotoxic activity of A-ring modified betulinic acid derivatives, Bioorg. Med. Chem. Lett. 13 (2003) 3137–3140;
(b) T. Honda, K.T. Liby, X. Su, C. Sundararajan, Y. Honda, N. Suh, R. Risingsong, C.R. Williams, D.B. Royce, M.B. Sporn, G.W. Gribble, Design, synthesis, and antiinflammatory activity both in vitro and in vivo of new betulinic acid analogues having an enone functionality in ring A, Bioorg. Med. Chem. Lett. 16 (2006) 6306–6309;
(c) K. Liby, T. Honda, C.R. Williams, R. Risingsong, D.B. Royce, N. Suh, A.T. Dinkova-Kostova, K.K. Stephenson, P. Talalay, C. Sundararajan, G.W. Gribble, M.B. Sporn, Novel semisynthetic analogues of betulinic acid with diverse cytoprotective, antiproliferative, and proapoptotic activities, Mol. Cancer Ther. 6 (2007) 2113–2119;
(d) S. Chintharlapalli, S. Papineni, S. Liu, I. Jutooru, G. Chadalapaka, S.-D. Cho, R.S. Murthy, Y. You, S. Safe, 2-cyano-lup-1-en-3-oxo-20-oic acid, a cyano derivative of betulinic acid, activates peroxisome proliferator-activated receptor gamma in colon and pancreatic cancer cells, Carcinogenesis 28 (2007) 2337–2346;
(e) A. Koohang, N.D. Majewski, E.L. Szotek, A.A. Mar, D.A. Eiznhamer, M.T. Flavin, Z.Q. Xu, Synthesis and cytotoxicity of 2-cyano-28-hydroxy-lup-1-en-3-ones, Bioorg. Med. Chem. Lett. 19 (2009) 2168–2171.
[20] K.T. Liby, M.M. Yore, M.B. Sporn, Triterpenoids and rexinoids as multifunctional agents for the prevention and treatment of cancer, Nat. Rev. Cancer 7 (2007) 357–369.
[21] (a) B.A. Shah, R. Kaur, P. Gupta, A. Kumar, V.K. Sethi, S.S. Andotra, J. Singh, A.K. Saxena, S.C. Taneja, Structure–activity relationship (SAR) of parthenin analogues with pro-apoptotic activity: development of novel anti-cancer leads, Bioorg. Med. Chem. Lett. 19 (2009) 4394–4398;
(b) A. Kumar, F. Malik, S. Bhushan, V.K. Sethi, A.K. Shahi, J. Kour, S.C. Taneja, G.N. Qazi, J. Singh, An essential oil and its major constituent isointermedeol induce apoptosis by increased expression of mitochondrial cytochrome c and apical death receptors in human leukaemia HL-60 cells, Chem. Biol. Interact. 171 (2008) 332–347;
(c) B.A. Shah, R. Chib, P. Gupta, V.K. Sethi, S. Koul, S.S. Andotra, A. Nargota, S. Sharma, A. Panday, S. Bani, B. Purnima, S.C. Taneja, Saponins as novel TNF-α inhibitors: isolation of saponins and a nor-pseudoguaianolide from *Parthenium hysterophorus*, Org. Biomol. Chem. 7 (2009) 3230–3235;
(d) B.A. Shah, S.C. Taneja, V.K. Sethi, P. Gupta, S.S. Andotra, S.S. Chimni, G.N. Qazi, The formation of novel 1,3- dioxolanes: atypical Baylis–Hillman reaction of a sesquiterpene lactone parthenin, Tetrahedron Lett. 48 (2007) 955–960.
[22] B.A. Shah, A. Kumar, P. Gupta, M. Sharma, V.K. Sethi, A.K. Saxena, J. Singh, G.N. Qazi, S.C. Taneja, Cytotoxic and apoptotic activities of novel amino analogues of boswellic acids, Bioorg. Med. Chem. Lett. 17 (2007) 6411–6416.

[23] G.S.R.S. Rao, P. Kondaiah, S.K. Singh, P. Ravanan, M.B. Sporn, Chemical modifications of natural triterpenes - glycyrrhetinic and boswellic acids: evaluation of their biological activity, Tetrahedron 64 (2008) 11541–11548.
[24] D.E. Fisher, Apoptosis in cancer therapy: crossing the threshold, Cell 78 (1994) 539–542.
[25] A.H. Wyllie, J.F. Kerr, A.R. Currie, Cell death: the significance of apoptosis, Int. Rev. Cytol. 68 (1980) 251–306.
[26] (a) S. Rello, J.C. Stockert, V. Moreno, A. Gamez, M. Pacheco, A. Juarranz, M. Canete, A. Villanueva, Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments, Apoptosis 10 (2005) 201–208;
(b) P.R. Sharma, D.M. Mondhe, M. Shanmugavel, H.C. Pal, A.K. Shahi, A.K. Saxena, G.N. Qazi, Anticancer activity of an essential oil from *Cymbopogon flexuosus*, Chem. Biol. Interact. 179 (2008) 160–168.
[27] S. Bhushan, J. Singh, M.J. Rao, A.K. Saxena, G.N. Qazi, A novel lignan composition from *Cedrus deodara* induces apoptosis and early nitric oxide generation in human leukemia Molt-4 and HL-60 cells, Nitric Oxide 14 (2006) 72–88.
[28] Z. Yu, W. Li, Induction of apoptosis by puerarin in colon cancer HT-29 cells, Cancer Lett. 238 (2006) 53–60.