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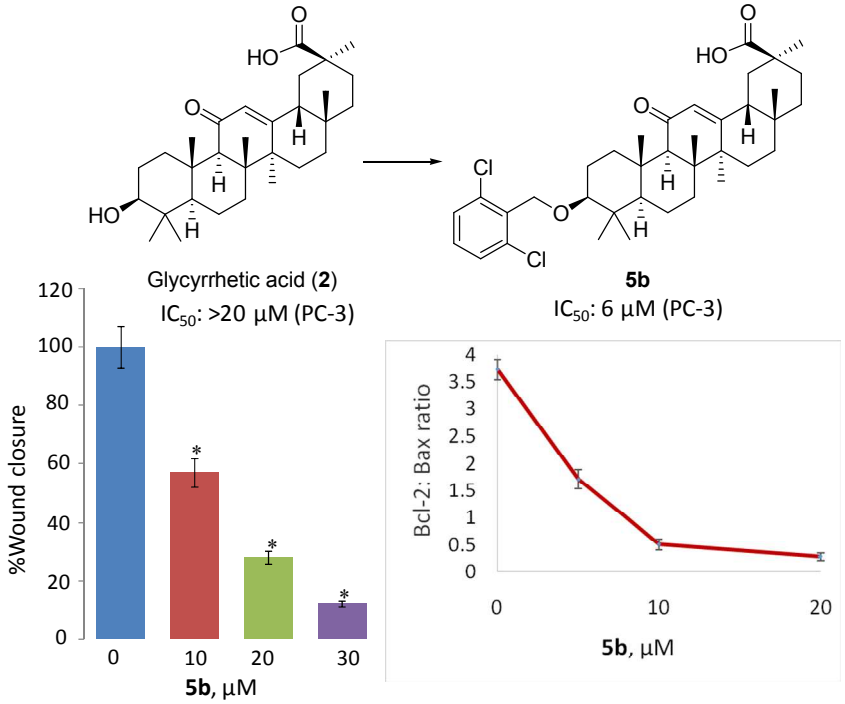
GRAPHICAL ABSTRACT



Synthesis and biological evaluation of semisynthetic analogs of glycyrrhetic acid is described.

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Concise Article

3-(2,6-Dichloro-benzyloxy)-11-oxo-olean-12-ene-29-oic acid, a semisynthetic derivative of glycyrrhetic acid: Synthesis, antiproliferative, apoptotic and anti-angiogenesis activity

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Glycyrrhetic acid (**2**, 3β-hydroxyl-11-oxo-olean-12-ene-29-oic acid), a pentacyclic triterpenoid isolated from *Glycyrrhiza glabra* is known to possess wide range of biological activities. Herein, we report synthesis and antiproliferative activity of 3-*O*-ether derivatives of glycyrrhetic acid. The cytotoxicity of prepared derivatives was investigated in three cancer cell lines, including human pancreatic (MIAPaCa-2), prostate (PC-3) and human hepatocellular liver carcinoma (HepG-2). Amongst tested compounds, the 2,6-dichlorobenzyl **5b** and 2,4-dichlorobenzyl derivative **5r** displayed significant cytotoxicity in PC-3 cells with IC_{50} values of 6 and 18 μM, respectively. The dichlorobenzyl derivative **5b** also displayed cytotoxicity in MIAPaCa-2 (IC_{50}: 7 μM) and HepG-2 cells (IC_{50}:19 μM). Further, the compound **5b** was investigated for apoptosis-induction by cell cycle analysis, nuclear morphological changes and mitochondrial membrane potential loss in PC-3 cells. Compoound **5b** led to increase in sub-G1 population in PC-3 cells, which is indicative of its apoptotic property. Interestingly, compound **5b** also arrested S-phase of the cell cycle. The nuclear morphology of PC-3 cells after treatment with compound **5b** was also investigated which confirmed the formation of apoptotic bodies. The compound **5b** induced apoptosis through both intrinsic and extrinsic apoptotic pathways in PC-3 cells, which was confirmed by mitochondrial membrane potential loss, inhibition of pro-caspase-3, 8 and 9 and cleavage of PARP-1. Furthermore, there was a significant decrease in Bcl-2/Bax ratio by compound **5b** in PC-3 cells. Interestingly, compound **5b** also inhibited the VEGF-induced PC-3 cell migration and decreased wound closure percentage from 100 to 12% at 30 μM. Similarly, compound **5b** inhibited angiogenesis-dependent cell migration in HUVEC cells and decreased wound closure from 100 to 20% at 30 μM, indicating its anti-angiogenic activity.

Introduction

Glycyrrhiza glabra (Licorice) is a tall shrub of the Leguminosae family, widely cultivated throughout Europe, the Middle East and Asia.[1] The ethnomedical use of *G. glabra* has been documented in several traditional systems of medicine. The rhizomes of licorice have been used worldwide as an herbal medicine and natural sweetener (30-50 times sweeter than sucrose).[2] *G. glabra* and its active components are reported to possess wide range of biological activities,[3] however the most active component which is responsible for its medicinal properties is a triterpene saponin glycyrrhizin (**1**, also called as glycyrrhizinic acid and glycyrrhizic acid).[3d] Glycyrrhizin also inhibits specific changes that occur in a cell under the action of the TPA (12-*O*-tetradecanoylphorbol-13-acetate; a tumor promotor),[4] and also suppressed estrogen-related endometrial cancer by inhibiting COX-2, IL-1α and TNF-α.[5]

Glycyrrhetic acid (**2**, 3β-hydroxyl-11-oxo-olean-12-ene-29-oic acid; also called as glycyrrhetinic acid), is a aglycone of glycyrrhizin, and is also present in *G. glabra*.[6] Glycyrrhetic acid belongs to the class of ursane-type pentacyclic triterpenoid and has wide range of biological activities including anti-inflammatory,[7] anti-ulcer, analgesic, anti-type IV allergic,[3d, 8] prevention of metabolic and vascular diseases[9] and anticancer activity.[3c, 9] The glycyrrhetic acid (**2**) is reported to possess cytotoxicity, and apoptosis-inducing activity in different hepatic, stomach, melanoma, breast and leukemia cancer cell lines.[10] It showed cytotoxic activity in HL-60 cells with IC_{50} of 63.2 μM.[11]

Glycyrrhetic acid also showed protection against UV-induced skin cancer,[12] and also increased natural killer cell activity in metastatic tumor.[13] Another interesting property of glycyrrhetic acid is chemosensitizing effect with various clinical oncology drugs. It partly reversed multidrug resistance (MDR) in Pgp-expressing cells (KB-C2) by increasing the intracellular accumulation of antitumor drugs.[14] Based on these findings, it is likely that glycyrrhetic acid derivatives elicit antitumor activity through multiple signaling pathways. In general, the cytotoxic or apoptotic activity of glycyrrhetic acid in most of the studies was

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moderate; thus research efforts were mainly focused on the identification of its derivatives with improved activity.[11b, 15] The structure-activity relationship of glycyrrhetic acid has been reviewed recently.[11b] The modifications on ring A were found to be more effective. A 2-trifluoromethyl derivative displayed potent cytotoxicity in 253JB-V (IC_{50}= 0.67 μM), KU7 (IC_{50}= 0.38 μM), PANC-1 (IC_{50}= 0.82 μM) and PANC-28 (IC_{50}= 1.14 μM) cell lines.[16] Glycyrrhetic acid derivative with C-3 alkoxyimino group and C-30 carboxylic acid methyl ester showed improvement in cytotoxicity in HL-60 cells from IC_{50} of 63 to 19 μM.[11a] Fused heterocyclic rings at C-2 and C-3 positions of glycyrrhetic acid led to improvement in cytotoxicity by 20-fold.[17] In general, the C-3 modifications were more successful, and led to identification of two antiulcer drugs carbenoxolone (**3**) and acetoxolone (**4**).[18] Carbenoxolone has also been reported to possess chemopreventive activity.[19] The literature precedence indicated that substitution of lipophilic groups at C-3 position is beneficial. Thus, herein we aimed to prepare new C-3 ether derivatives of glycyrrhetic acid and investigate their cytotoxicity in various cancer cell lines. Furthermore, the most promising cytotoxic compound was then mechanistically investigated in detail, in a panel of assays for apoptosis-inducing activity and anti-angiogenesis activity.

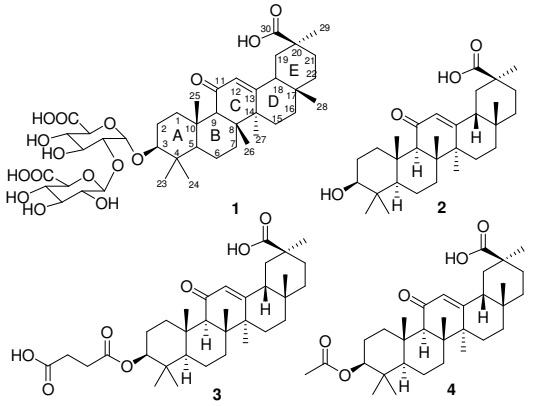
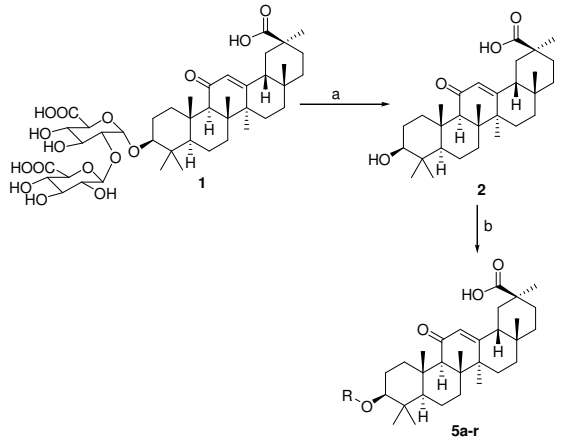


Figure 1. Chemical structures of glycyrrhizin (**1**), its aglycone glycyrrhetic acid (**2**) and known derivatives **3-4** of glycyrrhetic acid

Results and discussion

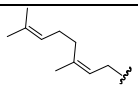
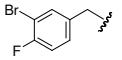
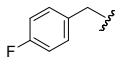
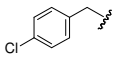
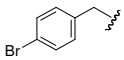
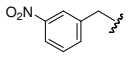
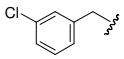
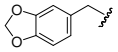
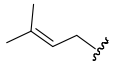
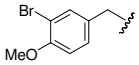
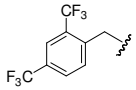
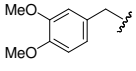
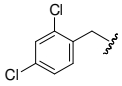
Glycyrrhizin (**1**) was isolated from *G. glabra* using reported procedure.[20] Aglycone **2** was then obtained by acid hydrolysis of glycyrrhizin (**1**). The C-3 etherification was achieved by adding different benzyl or alkyl chlorides in a solution of glycyrrhetic acid (**2**) in dry acetone, under alkaline conditions (Scheme 1).



Scheme 1. Synthesis of derivatives **5a-r** from glycyrrhizic acid (**1**). Reagents and conditions: (a) 5% Aq. HCl, MeOH, 90 °C, 20 h. (b) K_2CO_3 (1.2 equiv), R-Cl (for R, see Table 1, **5a** to **5r**) (1 equiv), dry acetone, 0 °C, 2 h, yield: see Table 1.

A series of ethers were synthesized and characterized by NMR and MS analysis. The list of synthesized derivatives is shown in Table 1.

Table 1. Synthesized glycyrrhetic acid derivatives

Entry	R	% yield	Entry	R	% yield
5a		92	**5j**		92
5b		95	**5k**		97
5c		90	**5l**		95
5d		88	**5m**		95
5e		95	**5n**		97
5f		94	**5o**		95
5g		90	**5p**		90
5h		90	**5q**		90
5i		85	**5r**		92

All compounds were evaluated for antiproliferative activity in three human cancer cell lines including pancreatic (MIAPaCa-2), prostate (PC-3) and hepatocellular carcinoma (HepG-2). Cytotoxicity results of most promising compounds are shown in Table 2. Results for all compounds are shown in ESI (Table S1).

Table 2. Cytotoxicity of most active compounds in different cancer cell lines [a,b,c,d]

Entry	IC_{50} (μM)		
	PC-3	MIAPaCa-2	HepG2
5a	10 ± 1.0	11 ± 0.97	>100
5b	6.0 ± 0.46	7.0 ± 0.56	19 ± 1.18
5c	9.0 ± 0.49	12 ± 1.0	>100
5r	18 ± 1.11	>100	>100
Paclitaxel	0.012 ± 0.012	0.1 ± 0.003	>100
Camptothecin	1.2 ± 0.067	0.19 ± 0.008	0.2 ± 0. 01

[a]Cells were grown in 96-well culture plates and treated with various concentrations of each test compounds for 48 h. Thereafter, cells were incubated with MTT solution for 2 h and the optical density of formazan crystals was measured as described in the experimental section. [b]HepG2, hepatocellular carcinoma cells; MIAPaCa-2, pancreatic cancer cells; PC-3, prostate cancer cells. [c]Compounds **2**, and **5d-5q** were inactive against all cell lines used (IC_{50} > 20 μM). [d] Data are Mean ± SD (n= 3).

All the tested compounds were found to be more sensitive against prostate cancer PC-3 cells in comparison to MIAPaCa-2 and HepG-2 cells. Compound **5b** (2,6-dichloro-benzyloxy derivative) showed significant cytotoxicity in all three tested cell lines and it caused concentration and time-dependent inhibition of PC-3 cell proliferation. It showed varying cytotoxicity potential (IC_{50}) at different time points as depicted in Figure 2. The main purpose of this experiment was to check the IC_{50} value of compound **5b** at 24 h time point, for the purpose of cell cycle, MMP loss, microscopy, wound scratch and western blot experiments.

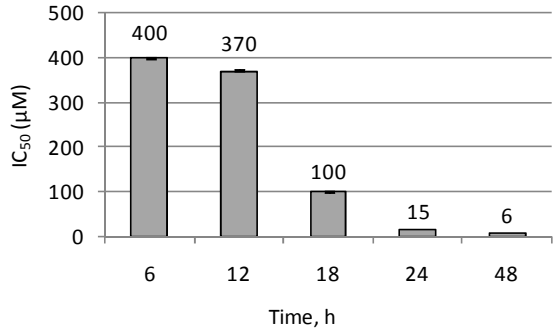


Figure 2. Cytotoxicity of compound **5b** in PC-3 cells at different time points. The cells were grown in 96-well culture plate and treated with different concentrations of compound **5b** for indicated time intervals. Cells were incubated with MTT solution and optical density of formazan crystals was measured as described in Materials and Methods. Data are Mean ± SD (n= 8 wells), and representative of three similar experiments.

In order to address the cell death caused by compound **5b**, the extent of apoptotic death in PC-3 cells was assessed using flow cytometry through determination of sub-G1 cell population by propidium iodide (PI) staining. As depicted in Figure 3, the regions marked with different colors represent % population at different phases of the cell cycle. PC-3 cells exposed to compound **5b** for 24 h exhibited a dose-dependent increase in sub-G1 fraction (<2n DNA), which may comprise both apoptosis and debris fraction implying together the extent of cell death (Figure 3). The sub-G1 apoptotic population was found to be 8, 12 and 37% following 5, 10 and 20 μM of **5b** treatment compared to control (untreated cells - 3%). Interestingly, compound **5b** significantly arrested the S-phase of the cell cycle in a dose-dependent manner in PC-3 cells, which ultimately results in blockage of cell (DNA) division.

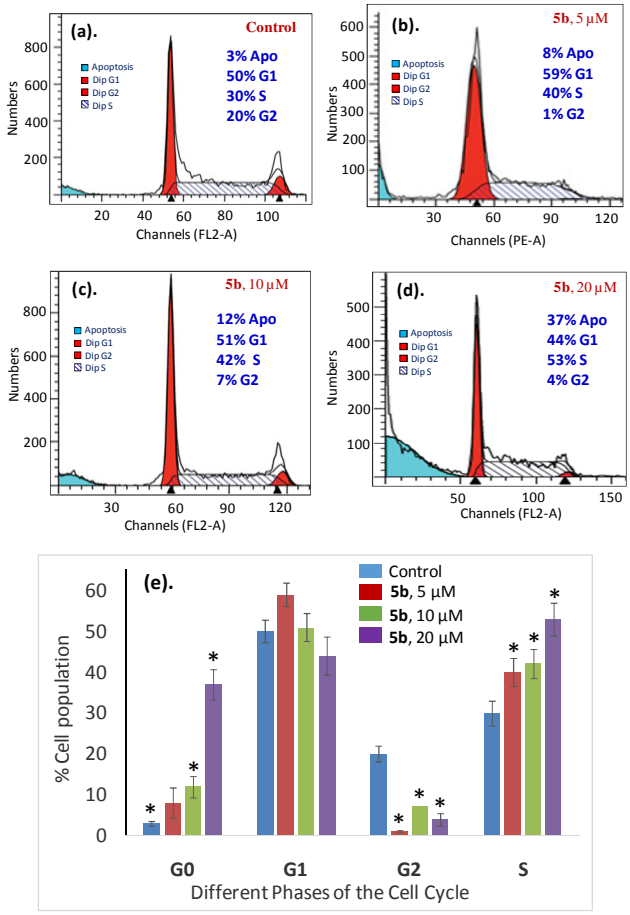


Figure 3. DNA cell cycle analyses in PC-3 cells exposed to compound **5b**. PC-3 cells were treated with different concentrations (5, 10 and 20 μM) of compound **5b** for 24 h and stained with Propidium iodide, PI (10 μg/ml) to determine DNA fluorescence and cell cycle phase distribution as described in Materials and Methods. Data were analyzed by Modfit software (Verity Software House Inc., Topsham, ME) for the proportions of different cell cycle phases. The fraction of cells from apoptosis (sub-G1/G0), G1, S and G2 phases analyzed from FL2- A vs. cell counts are shown in %. Data are representative of one of three similar experiments.

The apoptosis induction results obtained from cell cycle analysis were further corroborated by studying nuclear morphological changes of cells by fluorescence microscopy. After the treatment at 5, 10, and 20 μM of compound **5b**, characteristic changes of apoptosis such as nuclear condensation, membrane blebbing and

formation of apoptotic bodies were observed in the morphology of treated cells in a concentration-dependent manner, whereas untreated cells nuclei were found to be of normal intact morphology. The results suggest that compound **5b** was able to induce apoptotic cell morphology in PC-3 cells (Figure 4).

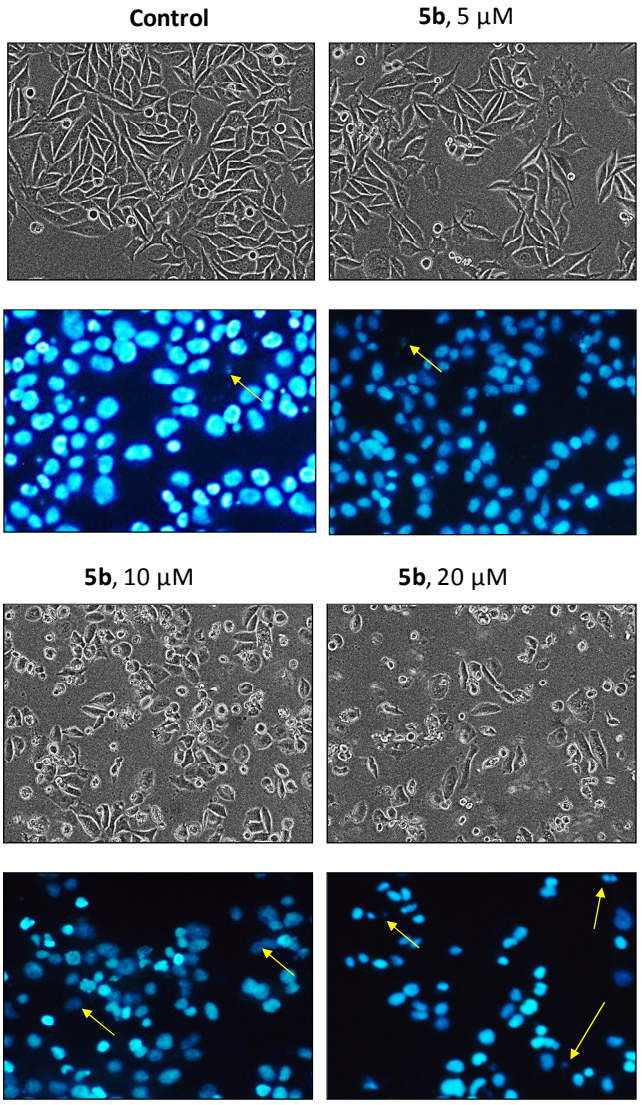


Figure 4. Effect of compound **5b** on cellular and nuclear morphology of PC-3 cells. Cells were treated with indicated concentrations of compound **5b** for 24 h time period and subsequently stained with Hoechst 33258 as described in experimental section and visualized for nuclear morphology and apoptotic bodies' formation. Data are representative of one of three similar experiments and magnification of the pictures was 30X on Olympus 1X 70 inverted microscopes.

Compound **5b** induced apoptosis through both intrinsic and extrinsic apoptotic pathways, which was confirmed by mitochondrial membrane potential (MMP) loss. Mitochondrial damage to cells results in perturbation of MMP. The loss in MMP ($\Delta\psi_m$) of PC-3 cells by compound **5b** was studied using rhodamine123 dye, which was reduced by healthy mitochondria into fluorescent probe whose fluorescence was measured by flow cytometer in FL-1 channel. In the untreated control cells, almost all cells were functionally active with high Rh-123 fluorescence. Mitochondrial damage results in decrease in Rh-123 Fluorescence. Compound **5b** at 10 µM caused mitochondrial damage and hence led to increase in the mitochondrial membrane potential loss by about 15%, which was further increased to 38% at 20 µM (Figure 5). The loss of mitochondrial membrane potential ($\Delta\psi_m$) is largely due to the opening of mitochondrial permeability transition pores (PTP), which conduit the leakage of proapoptoic proteins from mitochondria to cytosol.[21]

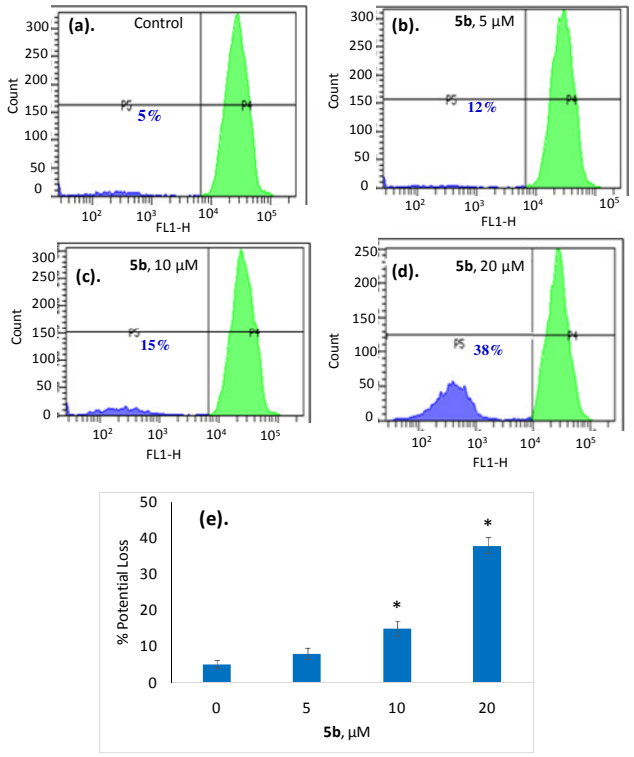


Figure 5 Compound **5b** induced mitochondrial membrane potential loss in prostate cancer PC-3 cells. Cells were treated with compound **5b** at 5, 10, and 20 µM concentration for 24 h time period. Cells were stained with Rhodamine-123 (200 nM) dye for 30 min and analyzed in FL-1 vs. counts channels of flow cytometer. Data are representative of one of three similar experiments at different time period.

Next, the effect of compound **5b** on key mitochondrial apoptotic proteins (pro-caspases and PARP-1) and Bcl-2/Bax ratio was investigated. The expression of anti-apoptotic protein Bcl-2 was significantly decreased by compound **5b** at 10 and 20 µM concentrations in PC-3 cells (Figure 6a). The downregulation of anti-apoptotic Bcl-2 protein caused the structural deformation of mitochondria, which opens mitochondrial permeability transition pores and release pro-apoptotic proteins to the cytosol and translocation of Bax from cytosol to mitochondria. Activation of Bax leads to cleavage of pro-caspase-9, activation of pro-caspase-3 and finally cleavage of downstream target poly(ADP-ribose) polymerase-1 (PARP-1). Compound **5b** was also found to inhibit the pro-caspase-8 expression in PC-3 cells. Hence, both intrinsic and extrinsic apoptotic pathways seemed to play a role in the

activation of executioner pro-caspase-3. The Bcl-2/Bax ratio in PC-3 cells was also determined. The compound **5b** drastically reduced the ratio from 5 to 0.5 (Figure 6b), indicating significant apoptotic behavior of the compound **5b**.

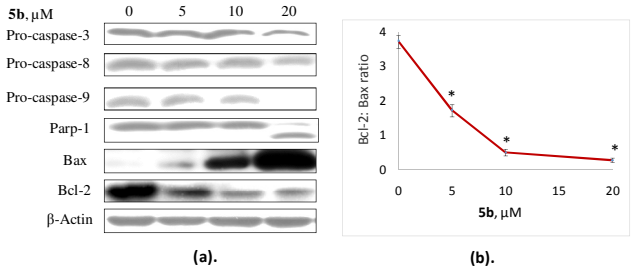


Figure 6. Influence of compound **5b** on the expression of important proteins involved in the initiation of apoptosis. PC-3 cells were treated with 5-20 µM concentrations of compound **5b** for 24h. Protein lysates were prepared and electrophoresis as described in Materials and Methods. β-actin was used as an internal control to represent the same amount of proteins applied for SDS-PAGE. Specific antibodies were used for detection of the indicated proteins in designated cell lysates. (a) Compound **5b** induced differential activation of different caspases, mitochondrial apoptotic proteins PARP-1 in PC-3 cells. Western blot analyses of the indicated proteins were performed in the whole cell lysate. Data are representative of one of three similar experiments. (b) Influence of compound **5b** on the Bcl-2/Bax ratio in PC-3 cells. Mitochondria and Bcl-2 family of proteins play a pivotal role in the induction of apoptosis.[21] Bcl-2 associated proteins have both pro-apoptotic and anti-apoptotic effects in cancer cells. These proteins regulate mitochondrial outer membrane potential and control the release of many apoptotic factors originating in the mitochondria. Compound 5-b decreases the expression of anti-apoptotic protein Bcl-2 and increase the expression of pro-apoptotic protein Bax in a dose dependent manner. The relative density of each band was measured as arbitrary units by Quantity One software of Bio-RAD gel documentation system. Data expressed as mean ± SD of three independent experiments.

Angiogenesis is one of the common hallmark manifestations of all cancers and it is an elementary event in the development of tumor growth and malignancy. To appraise the anti-angiogenic property of compound **5b** *in vitro*, the chemotactic motility of PC-3 cells was examined by wound-healing migration assay. Cell migration is necessary for tumor growth and metastasis. It was observed that compound **5b** significantly inhibited VEGF-induced HUVEC migration and decrease wound closure percentage from 100% to 12% at 20 µM concentrations (Figure 7a-b). The effect of compound **5b** was also investigated in angiogenesis dependent cell migration in HUVEC cells. Results are shown in Figure 7c-d, which indicated that compound **5b** inhibited angiogenesis-dependent cell migration in HUVEC cells in a dose-dependent manner. The significant % wound closure was observed at 10 and 20 µM of **5b** treatment.

Conclusion

In conclusion, the semisynthetic derivative **5b** displayed significant antiproliferative activity in human prostate PC-3 cells. Mechanistic study revealed that, derivative **5b** also possesses potent apoptotic and anti-angiogenesis potential in PC-3 cells. It induces apoptosis which is confirmed by apoptotic bodies' formation, increase of sub-G1 population and induction of various pro-apoptotic proteins. Compound **5b** caused disruption of mitochondrial membrane potential, rendered Bcl-2 inhibition, Bax translocation, decrease Bcl-2/Bax ratio, and released pro-apoptotic factors from the mitochondria. Mitochondria and Bcl-2 family of proteins play a pivotal role in the induction of apoptosis. The cell death regulated by Bcl-2 associated proteins have both pro-apoptotic and anti-apoptotic effects in cancer cells. These proteins regulate mitochondrial outer membrane potential and control the release of many apoptotic factors originating in the mitochondria. These events are accompanied by activation of pro-caspases-3, 8, -9, which cleave PARP-1 and finally induce apoptosis. Compound **5b** also inhibit pro-caspase-8 level, therefore it induces apoptosis through both intrinsic and extrinsic apoptotic pathways. These results provide the basis for further in-depth drug targeted studies, while the pro-apoptotic feature of compound **5b** raises its potential as a future anticancer therapeutics.

Experimental Section

General. All chemicals were obtained from Sigma-Aldrich Company and used as received. ^{1}H, ^{13}C and DEPT NMR spectra were recorded on Bruker-Avance DPX FT-NMR 500 and 400 MHz instruments. Chemical data for protons are reported in parts per million (ppm) downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent ($CDCl_3$, 7.26 ppm). Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 125 MHz or 100 MHz: chemical data for carbons are reported in parts per million (ppm, δ scale) downfield from tetramethylsilane and are referenced to the carbon resonance of the solvents ($CDCl_3$: 77.36 ppm). ESI-MS and HRMS spectra were recorded on Agilent 1100 LC-Q-TOF and HRMS-6540-UHD machines. IR spectra were recorded on Perkin-Elmer IR spectrophotometer. Melting points were recorded on digital melting point apparatus. HPLC analysis was done on Waters and Shimadzu HPLC systems (detailed method provided in ESI).

Plant material. The dried *Glycyrrhiza glabra* roots were purchased from the local market of Jammu and it was authenticated by B. K. Kapahi, Biodiversity and Applied Botany Division of Indian Institute of Integrative Medicine (CSIR), Jammu. A specimen sample (accession number: 13485) was preserved in Janaki Ammal Herbarium at the CSIR-IIIM, Jammu, India.

Isolation of glycyrrhizin. *G. glabra* roots were extracted with water: ethanol (8: 2) under warm condition (55-60 °C). The crude extract was filtered and concentrated, which was washed with cold dichloromethane and cold acetone to remove other low molecular weight phenolics. By repeating this procedure glyccyrrhizin-enriched fraction (80% glyccyrrhizin) was obtained. This material was directly used for further hydrolysis.[22]

Hydrolysis of glycyrrhizin. The glycyrrhizin was hydrolyzed by 5% aq. HCl to get glycyrrhetic acid, which was purified over

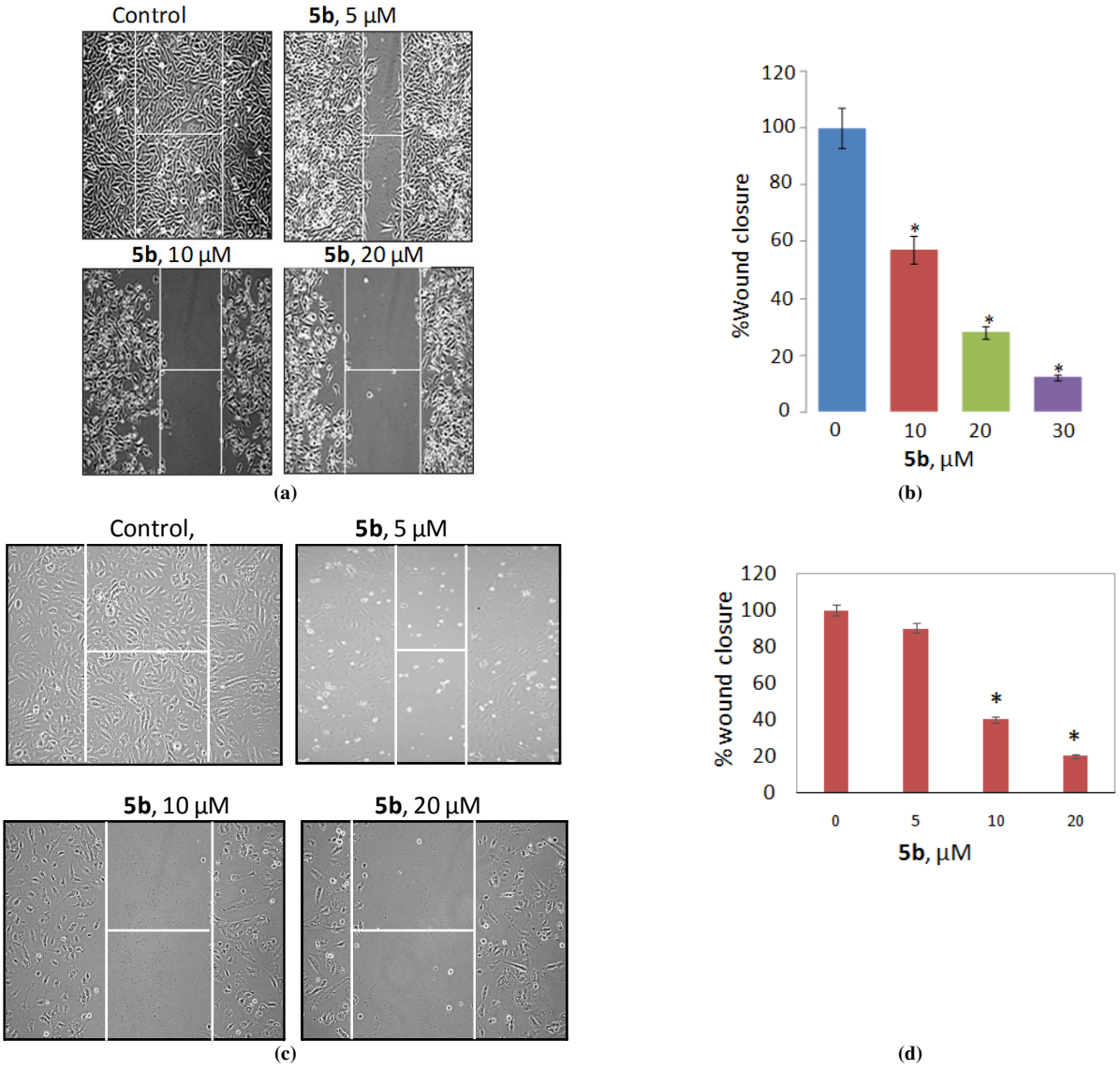


Figure 7. (a-b). Effect of compound **5b** on VEGF arbitrated *in vitro* angiogenesis in PC-3 cells. Compound **5b** inhibits PC-3 migration in the wound healing assay. PC-3 cells were scratched by a sterile micro tip and the areas were quantified in three random fields in the terms of wound closure percentage. **(c-d).** Effect of compound **5b** on angiogenesis-dependent cell migration in HUVEC cells. Data were mean ± S.D. of three independent experiments.

silica column in EtOAc: hexane – 25: 75. Pure glycyrrhetic acid was characterized by comparison of m.p. and spectral data with literature values.[6] The glycyrrhetic acid was then used for modifications at C-3 position for the synthesis of *O*-alkylated/benzylated glycyrrhetic acid derivatives.

General method for preparation of *O*-alkylated/benzylated glycyrrhetic acid (3β-hydroxy-11-oxoolean-12-en-30-olic acid). To the solution of 3β-hydroxy-11-oxoolean-12-en-30-olic acid (**2**, 100 mg, 1 mmol) in dry acetone (5 mL) was added K_2CO_3 (1.2 mmol) followed by addition of different alkyl and benzyl halides (1 mmol). The mixture was stirred at room temperature for 8 h under inert atmosphere and concentrated under reduced pressure. The reaction mixture was diluted with chloroform. Water was added to the resultant mixture leading to formation of a white precipitate in the aqueous layer. The organic layer was decanted off and the remaining solid residue was washed 5-6 times with chloroform. Combined chloroform layer was evaporated under reduced pressure and the residue obtained was purified by silica gel (#100-200) column chromatography using hexane-ethyl acetate as a eluent to yield the different alkylated products **5a-r**.

3-(2-Chloro-6-fluoro-benzyloxy)-11-oxo-olean-12-ene-29-oic acid (5a): white solid; HPLC: t_R = 11.9 min (100% purity); yield: 92%; m.p. 236-238 °C; IR ($CHCl_3$): ν_{max} 3844, 3900, 3400, 2852, 2923, 1731, 1657, 1609, 1582, 1385, 1259, 1311, 1259, 1209, 1153, 1083, 1039 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.39-7.29 (m, 3H, Ar-4´,5´,6´), 5.47 (s, 1H, CH-12), 5.32-5.47 (m, 2H, CH_2-1´), 3.20 (brs,1H, CH-3), 2.74 (tt, 1H, CH-18), 2.28 (s, 1H, CH-8), 2.05 (m, 2H, CH-2), 2.01 (m, 2H, CH-1), 1.32 (s, 3H, Me-27), 1.17(s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.11 (s,

3H, Me-26), 0.99 (s, 3H, Me-23), 0.80 (s, 3H, Me-24), 0.80 (s, 3H, Me-28), 0.69 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.9 (C-11), 176.0 (C-30), 169.0 (C-13), 136.8 (C-3´, 7´), 131.4 (C-2´), 130.6 (C-5´), 128.5 (C-4´, 6´, 12), 78.7 (C-3), 61.7 (C-9), 61.7 (C-9), 54.8 (C-5), 48.2 (C-18), 45.3 (C-14), 44.3 (C-20), 43.1 (C-8), 40.9 (C-19), 39.1 (C-1, C-4), 37.6 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 31.1 (C-21), 28.4 (C-29), 28.4 (C-28), 28.3 (C-23), 27.2(C-2), 26.4 (C-15), 26.3 (C-16), 23.2 (C-27), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 613.3459 $[M+H]^+$ calcd for $C_{37}H_{50}ClFO_4 + H^+$ (613.3454).

3-(2,6-Dichloro-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5b): white solid; HPLC: t_R = 26.7 min (97% purity); yield: 95%; m.p. 226-228 °C; IR ($CHCl_3$): ν_{max} 3441, 2926, 2865, 1730, 1655, 1565, 1582, 1461, 1438, 1280, 1255, 1209, 1151, 1039, 1082 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.39 (d, 1H, Ar-4´), 7.37 (d, 1H, Ar-6´), 7.27 (m, 1H, Ar-5´), 5.42 (s, 1H, CH-12), 5.32-5.47 (m, 2H, CH_2-1´), 3.22 (dd, *J* = 4.0, 12.0 Hz, 1H, CH-3), 2.77 (tt, 1H, CH-18), 2.01 (m, 2H, CH-2),1.98 (m, 2H, CH-1), 1.32 (s, 3H, Me-27), 1.17 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.11 (s, 3H, Me-26), 0.99 (s, 3H, Me-23), 0.80 (s, 3H, Me-24), 0.80 (s, 3H, Me-28), 0.69 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.1 (C-11), 176.1 (C-30), 168.9 (C-13), 136.8(C-3´,7´), 131.5 (C-2´), 130.7 (C-5´), 128.5 (C-4´, 6´, C-12), 78.8 (C-3), 61.8 C-9), 61.3 (C-1´), 54.2 (C-5), 48.2 (C-18), 45.3 (C-14), 44.3 (C-20), 43.1 (C-8), 40.9 (C-19), 39.1 (C-1, C-4), 37.6 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 31.1 (C-21), 28.4 (C-29), 28.3 (C-28), 28.0 (C-23), 27.3 (C-2), 26.5 (C-15), 26.4 (C-16), 23.3 (C-27), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.5(C-24). HR-ESIMS: *m/z* 629.3161 $[M+H]^+$ calcd for $C_{37}H_{50}Cl_2O_4 + H^+$ (629.3158).

3-(4-Fluoro-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5c): white solid; HPLC: t_R = 8.4 min (96% purity); yield: 90%; m.p. 238-240 °C; IR ($CHCl_3$): ν_{max} 3790, 3663, 3436, 2922, 2853, 1730, 1650, 1579, 1617, 1513, 1345, 1386, 1084, 1215, 1260 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.36 (m, 2H, Ar-3´,7´), 7.08 (m, 2H, Ar-4´,6´), 5.56 (s, 1H, CH-12), 5.10-5.12 (dd, *J* = 12 Hz, 2H, CH_2-1´), 3.23 (dd, *J* = 4.0, 8.0 Hz,1H, CH-3), 2.81 (tt, 1H, CH-18), 2.32 (s, 1H, CH-9), 1.35 (s, 3H, Me-27), 1.14 (s, 3H, Me-26), 1.14 (s, 3H, Me-29), 1.11 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.81 (s, 3H, Me-24), 0.73 (s, 3H, Me-28), 0.71 (s, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.1 (C-11), 176.1 (C-30), 169.0 (C-13), 163.9 (d, $^1J_{CF}$ = 246 Hz, C-5´), 132.0 (C-2´), 130.3 (d, $^3J_{CF}$ = 9 Hz, C-3´, C-7´), 128.5 (C-12), 115.6 (d, $^2J_{CF}$ = 21 Hz, C-4´, C-6´), 78.5 (C-3), 65.4 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 43.9 (C-20) 43.1 (C-8), 41.0 (C-19), 39.1 (C-1, C-4), 37.6 (C-22), 37.0 (C-10), 32.7 (C-7), 31.7 (C-17), 31.1 (C-21), 28.4 (C-29), 28.2 (C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.3 (C-16), 23.3 (C-23), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 579.3846 $[M+H]^+$ calcd for $C_{37}H_{51}FO_4 + H^+$ (579.3844).

3-(2-Iodo-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5d): white solid; HPLC: t_R = 48.4 min (98% purity) yield: 88%; m.p. 228-230 °C; IR ($CHCl_3$): ν_{max} 3435, 2923, 2852, 1729, 1656, 1463, 1384, 1311, 1259, 1210, 1150, 1045, 1084 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, 1H, Ar-4´), 7.33 (m, 2H, Ar-5´, 7´), 6.96 (m, 1H, Ar-6´), 5.51 (s, 1H, CH-12), 5.10 (s, 2H, CH_2-1´), 3.16 (d, *J* = 4.0, 12.0 Hz, 1H, CH-3), 2.73 (tt, 1H, CH-18), 2.25 (s, 1H, CH-9), 1.18 (s, 3H, Me-27), 1.13 (s, 3H, Me-25), 1.06 (s, 3H, Me-29), 1.04 (s, 3H, Me-26), 0.93 (s, 3H, Me-23), 0.73 (s, 3H, Me-24), 0.71 (s, 3H, Me-28), 0.63 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ 200.1 (C-11), 175.8 (C-30), 168.4 (C-13), 139.6 (C-2´), 138.5 (C-4´), 130.3 (C-5´), 129.7 (C-7´), 128.6 (C-12), 120.5 (C-6´), 78.8 (C-3), 70.1 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 44.1 (C-20), 43.1 (C-8), 41.1 (C-19), 39.1 (C-1, C-4), 37.7 (C-22), 37.1 (C-10), 32.8 (C-7), 31.8 (C-17), 31.2 (C-21), 28.4 (C-29), 28.1 (C-23, C-28), 27.5 (C-2), 26.4 (C-15), 26.2 (C-16) , 23.3 (C-23), 18.7 (C-26), 17.5 (C-6), 16.3 (C-25), 15.5 (C-24); HR-ESIMS 687.2917 $[M+H]^+$ calcd for $C_{37}H_{51}IO_4 + H^+$ (687.2904).

3-(3-Nitro-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5e): Cream solid; HPLC: t_R = 49.6 min (90% purity); yield: 95%; m.p. 186-188 °C; IR ($CHCl_3$): ν_{max} 3448, 3053, 2928, 2868, 2304, 1730, 1656, 1619, 1585, 1462, 1386, 1327, 1313, 12679, 1262, 1210, 1149, 1039 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (d, 1H, Ar-3´, 5´), 7.71 (m, 2H, Ar-7´), 7.59 (m, 1H, Ar-6´), 5.55 (s, 1H, CH-12), 5.24 (m, 2H, CH_2-1´), 3.23 (dd, *J* = 4.0, 8.0 Hz,1H, CH-3), 2.80 (tt, 1H, CH-18), 2.33 (s, 1H, CH-9), 1.36 (s, 3H, Me-27), 1.19 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.11 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.80 (s, 3H, Me-24), 0.76 (s, 3H, Me-28), 0.71 (dd, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.4 (C-11), 176.1 (C-30), 168.9 (C-13), 148.7 (C-4), 138.6 (C-2´), 134.2 (C-7´), 130.1 (C-6´), 128.9 (C-12), 123.6 (C-3´), 123.1 (C-5´), 79.1 (C-3), 65.1 (C-1´), 62.1 (C-9), 55.2 (C-5), 48.6 (C-18), 45.7 (C-14), 44.4 (C-20), 43.5 (C-8), 41.4 (C-19), 39.9 (C-1,C-4), 38.0 (C-22), 37.4 (C-10), 33.0 (C-7), 31.4 (C-17), 31.4 (C-21), 28.8 (C-29), 28.6 (C-28), 28.4 (C-23), 27.6 (C-2), 26.9 (C-15), 26.7 (C-16), 23.7 (C-27), 18.9 (C-26), 17.5 (C-6), 16.7 (C-25), 15.9 (C-24); HR-ESIMS: *m/z* 606.3786 $[M+H]^+$ calcd for $C_{37}H_{51}N\,O_6 + H^+$ (606.3789).

3-(2-Nitro-benzyloxy)-11-oxo-olean-12-ene-29-oic acid (5f): white solid; HPLC: t_R = 7.8 min (100% purity); yield: 94%; m.p.186-188 °C; IR ($CHCl_3$): ν_{max} 3448, 3053, 2928, 2868, 2304, 1730, 1656, 1619, 1585, 1462, 1386, 1327, 1313, 12679, 1262, 1210, 1141, 1039 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 8.05 (d, 1H, Ar-3´), 7.63 (m, 1H, Ar-5´), 7.61 (m, 1H, Ar-6´), 7.45 (m, 1H, Ar-4´), 5.51 (s, 1H, CH-12), 5.43 (m, 2H, CH_2-1´), 3.16 (dd, *J* = 8.0, 12.0 Hz, 1H, CH-3), 2.70 (tt, 1H, CH-18), 2.26 (s, 1H, CH-9), 1.91 (m, 2H, CH-2), 1.20 (s, 3H, Me-27), 1.18 (s, 3H, Me-25), 1.14 (s, 3H, Me-29), 1.14 (s, 3H, Me-26), 1.04 (s, 3H, Me-23), 0.93 (s, 3H, Me-24), 0.93 (s, 3H, Me-28), 0.73 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.7 (C-11), 175.7 (C-30), 168.9 (C-13), 148.7 (C-3´), 133.8 (C-6´), 132.0 (C-2´), 129.3 (C-7´), 129.0 (C-5´), 128.6 (C-4´), 125.1 (C-12), 78.7 (C-3), 63.1 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.3 (C-18), 45.3 (C-14), 44.2 (C-20), 43.2 (C-8), 41.0 (C-19), 39.1 (C-1,C-4), 37.6 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 31.1 (C-21), 28.4 (C-29), 28.4 (C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.3 (C-16), 23.4 (C-27), 18.6 (C-26), 17.4 (C-6), 16.4 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 606.3802 $[M+H]^+$ calcd for $C_{37}H_{51}NO_6 + H^+$ (606.3789).

3-(3-Methyl-but-2-enyloxy)-11-oxo-olean-12-ene-29-oic acid (5g): cream colored oil; HPLC: t_R = 48.4 min (98% purity); yield: 90%; IR ($CHCl_3$): ν_{max} 3441, 2927, 2867, 1724, 1658,

1658, 1619, 1454, 1385, 1327, 1310, 1257, 1210, 1152, 1084, 1046 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 5.63 (s, 1H, CH-12), 5.34 (t, 1H, CH-2´), 4.62 (m, 2H, CH_2-1´), 3.23 (dd,1H, CH-2), 2.81 (tt, 1H, CH-18), 2.34 (s, 1H, CH-9), 1.77 (s, 3H, CH-3´), 1.72 (s, 3H, CH-4´), 1.36 (s, 3H, Me-27), 1.26 (s, 3H, Me-25), 1.14 (s, 3H, Me-29), 1.13 (s, 3H, Me-26), 1.01(s, 3H, Me-23), 0.81 (s, 3H, Me-24), 0.80 (s, 3H, Me-28) , 0.71 (d, 1H, CH-5), ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.2 (C-11), 176.4 (C-30), 169.3 (C-13), 128.4 (C-3´), 118.6 (C-2´), 78.7 (C-3´), 61.8 (C-1´), 61.2 (C-9), 54.9 (C-5), 48.3 (C-18), 45.3 (C-14), 44.2 (C-20),43.9 (C-2) , 43.2 (C-8), 41.1 (C-19), 39.1 (C-1, C-4), 37.7 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 29.7 (C-21), 28.5 (C-29), 28.3 (C-28), 28.1 (C-23), 27.3 (C-2), 26.5 (C-15), 26.4 (C-16), 25.7 (C-5´), 23.3 (C-27), 18.1 (C-26), 17.5 (C-6), 16.6 (C-4´), 16.4 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 539.4106 $[M+H]^+$ calcd for $C_{35}H_{54}O_4 + H^+$ (539.4094).

3-(2,4-Bis-trifluoromethyl-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5h): white solid; HPLC: t_R = 42.4 min (98% purity); yield: 90%; m.p. 254-256 °C; IR ($CHCl_3$): ν_{max} 3448, 3053, 2928, 2868, 2304, 1730, 1656, 1619, 1585, 1462, 1386, 1327, 1313, 12679, 1262, 1210, 1149, 1039 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.95 (d, 1H, Ar-4´), 7.87 (d, 1H, *J* = 8 Hz, Ar-6´), 7.72 (s, 1H, *J* = 8 Hz, Ar-7´), 5.60 (s, 1H, CH-12), 5.36 (m, 2H, CH_2-1′), 3.23 (dd, *J* = 8.0, 12.0 Hz 1H, CH-3), 2.80 (tt, 1H, CH-18), 2.33 (s, 1H, CH-9) 1.35 (s, 3H, Me-27), 1.21 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.12 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.81 (s, 1H, Me-24), 0.80 (s, 3H, Me-28), 0.71 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.1 (C-11), 175.7 (C-30), 168.7 (C-13), 138.5 (C-2´), 132.3 (C-5´), 130.1 (C-3´), 129.3 (C-7´, 129.0 (C-4´), 128.6 (C-12), 123.8 (CF_3), 123.4 (CF_3) 78.5 (C-3), 61.9 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 43.9 (C-20), 41.0 (C-19), 39.1 (C-1, C-4), 37.7 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 31.0 (C-21), 28.4 (C-29), 28.3 (C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.3 (C-16), 23.4 (C-27), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 697.3702 $[M+H]^+$ calcd for $C_{39}H_{50}F_{6,}O_4 + H^+$ (697.3686).

3-Methoxy-11-oxo-olean-12-ene-29-oic acid (5i): white solid; ; HPLC: t_R = 49.6 min (95%); yield: 85%; m.p. 262-264 °C; IR ($CHCl_3$): ν_{max} 3340, 2931, 2869, 1722, 1657, 1618, 1465, 1386, 1358, 1323, 1246, 1189, 1086, 1040 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 5.59 (s, 1H, CH-12), 3.62 (s, 3H, Me-1´), 3.16 (dd, *J* = 8.0, 12.0 Hz, 1H, CH-3), 2.74 (tt, 1H, CH-18), 2.27 (s, 1H, CH-9), 1.55 (m, 2H, CH-2), 1.18 (s, 3H, Me-27), 1.08 (s, 3H, Me-25), 1.08 (s, 3H, Me-29), 1.07 (s, 3H, Me-26), 1.06 (s, 3H, Me-23), 0.94 (s, 1H, Me-24), 0.93 (s, 3H, Me-28), 0.74 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.3 (C-11), 177.0 (C-30), 169.3 (C-13), 128.5 (C-12), 78.8 (C-3), 61.8 (C-9), 54.9 (C-5), 51.8 (C-1´), 48.4 (C-18), 45.4 (C-14), 44.0 (C-20), 43.2 (C-8), 41.0 (C-19), 39.1 (C-1, C-4), 37.7 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 31.1 (C-21), 28.5 (C-29), 28.3 (C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.4 (C-16), 23.4 (C-27), 18.7 (C-26), 17.4 (C-6), 16.4 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 485.3616 $[M+H]^+$ calcd for $C_{31}H_{48}O_4 + H^+$ (485.3625).

3-(3,7-Dimethyl-octa-2,6-dienyloxy)- 11-oxo-olean-12-ene-29-oic acid (5j): yellow oil; HPLC: t_R = 8.0 min (100% purity); yield: 92%; IR ($CHCl_3$): ν_{max} 3442, 2925, 2854, 1725, 1660, 1620, 1454, 1385 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 5.57 (s, 1H, CH-12), 5.27 (t, 1H, CH-2˙), 5.01 (t, 1H, CH-6˙), 4.54 (m, 2H, CH-1˙), 3.16 (dd, *J* = 8.0, 12.0 Hz, 1H, CH-3), 2.74 (tt, 1H, CH-18), 2.26 (s, 1H, CH-9), 1.65 (s, 3H, Me-8´), 1.60 (s, 3H, Me-9´), 1.52 (s, 3H, Me-10´), 1.27 (s, 3H, Me-27), 1.18 (s, 3H, Me-25), 1.07 (s, 3H, Me-29), 1.06 (s, 3H, Me-26), 0.93 (s, 3H, Me-23), 0.73 (s, 1H, Me-24), 0.73 (s, 3H, Me-28), 0.71 (d, 1H, CH-5);^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.2 (C-11), 179.4 (C-30), 169.3 (C-13),142.4 (C-7´), 131.7 (C-3´), 128.5 (C-12), 123.7 (C-6´), 118.4 (C-2´), 78.7 (C-3), 61.8 (C-9), 61.2 (C-1´), 54.9 (C-5), 48.3 (C-18), 45.3 (C-14), 43.9 (C-20), 43.1 (C-8), 41.1 (C-19), 39.5 (C-4´), 39.1 (C-1, C-4), 37.7 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 31.2 (C-21), 29.7 (C-5´)28.5 (C-29), 28.3 (C-28), 28.1 (C-27), 27.3 (C-2), 26.4 (C-9´), 26.4 (C-15), 26.3 (C-16), 25.7 (C- 10´), 23.3 (C-23), 18.6 (C-26), 17.4 (C-6), 16.5 (C-8´), 16.3 (C-25), 15.6 (C-24); HR-ESIMS: *m/z* 607.4734 $[M+H]^+$ calcd for $C_{40}H_{62}O_4H^+$ (607.4720).

3-(3-Bromo-4-fluoro-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5k): white solid; HPLC: t_R = 10.1 min (100% purity); yield: 97%; m.p. 298-299 °C; IR ($CHCl_3$): ν_{max} 3400, 2923, 2852, 1729, 1655, 1498, 1463, 1385, 1248, 1209, 1151, 1083, 1047 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.56 (dd, 1H, Ar-3´), 7.29 (m, 1H, Ar-7´), 7.13 (t, 1H, Ar-6´), 5.58 (s, 1H, CH-12), 5.08 (m, 2H, CH_2-1´), 3.23 (dd, *J* = 8.0, 12.0 Hz, 1H, CH-3), 2.79 (tt, 1H, CH-18), 2.33 (s, 1H, CH-9), 1.92 (dd, 2H, CH-2), 1.82 (dd, 2H, CH-1),1.35 (s, 3H, Me-27), 1.15 (s, 3H, Me-25), 1.14 (s, 3H, Me-29), 1.12 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.81 (s, 1H, Me-24), 0.75 (s, 3H, Me-28), 0.68 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.1 (C-11), 176.0 (C-30), 168.9 (C-13), 159.8 (d, $^1J_{CF}$ = 198 Hz, C-5´), 133.7 (d, $^3J_{CF}$ = 3 Hz, C-3´), 133.5 (C-11), 129.0 (d, $^2J_{CF}$ = 6 Hz, C-6´),128.5 (C-7´), 116.7 (C-12), 109.2 (d, $^2J_{CF}$ = 17 Hz, C-4´), 78.7 (C-3), 64.6 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.3 (C-18), 45.3 (C-14), 44.0 (C-20), 43.1 (C-8), 41.1 (C-19), 39.1 (C-4), 39.1 (C-1), 37.6 (C-22), 37.0 (C-4), 32.7 (C-7), 31.7 (C-10), 31.1 (C-21), 29.7 (C-5), 28.4 (C-29), 28.2 (C-28), 28.1 (C-23), 27.2 (C-2), 26.4 (C-15), 26.3 (C-16), 23.3 (C-27), 18.6 (C-26), 17.4 (C-6), 16.4 (C-25), 15.6 (C-24); HR-ESIMS: *m/z* 657.2944 ($M+H^+$) calcd for $C_{37}H_{50}BrF_{,}O_4 + H^+$(657.2949).

3-(4-Chloro-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5l): cream colored sticky solid; HPLC: t_R = 12.1 min (100% purity) yield: 95%; m.p. 260-261 °C, IR ($CHCl_3$): ν_{max} 3400, 2923, 2852, 1729, 1656, 1463, 1463, 1385, 1256, 1210, 1151, 1084, 1018 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.36-7.27 (m, 4H, Ar-3´,4´,6´,7´), 5.59 (s, 1H, CH-12), 5.10 (m, 2H, CH_2-1´), 3.23 (dd, *J* = 4.0, 8.0 Hz, 1H, CH-3), 2.78 (tt, 1H, CH-18), 2.33 (s, 1H, CH-9), 1.98 (dd, 2H, CH-2), 1.35 (s, 3H, Me-27), 1.15 (s, 3H, Me-25), 1.14 (s, 3H, Me-29), 1.12 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.98 (s, 1H, Me-24), 0.81 (s, 3H, Me-28), 0.71 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.2 (C-11), 176.1 (C-30), 169.0 (C-13), 134.6 (C-5´), 134.5 (C-2´), 129.6 (C-3´,7´), 128.8 (C-4´, C-6´), 128.4 (C-12), 78.7 (C-3), 65.4 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 44.0 (C-20), 43.1 (C-8), 41.0 (C-19), 39.1 (C-1, C-17), 37.6 (C-4), 37.7 (C-22), 32.7 (C-7), 31.7 (C-10), 31.1 (C-21), 28.4 (C-29), 28.2 (C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.3 (C-16), 23.3 (C-

27), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 595.3547 $[M+H]^+$ calcd for $C_{37}H_{51}ClO_4$ + H^+ (595.3548).

3-(4-Bromo-benzyloxy)- 11-oxo-olean-12-ene-29-oic acid (5m): colorless oil; HPLC: t_R = 12.7 min (95% purity); yield: 95%; IR ($CHCl_3$): ν_{max} 3411, 2925, 2855, 1729, 1657, 1488, 1455, 1385, 1279, 1256, 1210, 1150, 1083, 1071, 1014 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.51-7.49 (m, 2H, Ar-3´,4´), 7.22-7.24 (m, 2H, Ar-6´,7´), 5.59 (s, 1H, CH-12), 5.09 (m, 2H, CH_2-1´), 3.23 (dd, *J* = 8.0, 12.0 Hz, 1H, CH-3), 2.78 (tt, 1H, CH-18), 2.33 (s, 1H, CH-9), 2.01 (dd, 2H, CH-2),1.65 (dd, 2H, CH-1), 1.35 (s, 3H, Me-27), 1.15 (s, 3H, Me-25), 1.14 (s, 3H, Me-29), 1.12 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.98 (s, 1H, Me-24), 0.81 (s, 3H, Me-28), 0.75 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.2 (C-11), 176.1 (C-30), 168.9 (C-13), 135.1 (C-2´), 131.7 (C-4´, C-6´), 129.9(C-3´, C-7´), 128.5 (C-12), 78.7 (C-3), 65.4 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 44.0 (C-20), 43.2 (C-8), 41.0 (C-19), 39.1 (C-1, C-4), 37.8 (C-22), 37.1 (C-10), 32.7 (C-7), 31.7 (C-17), 31.1 (C-21), 28.4 (C-29, C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.3 (C-16), 23.4 (C-27), 18.6 (C-26), 17.4 (C-6), 16.4 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 639.3048 and 641.3035 $[M+H]^+$ calcd for $C_{37}H_{51}BrO_4 + H^+$ (639.3045 and 641.3029).

3-(3-Chloro-benzyloxy)-11-oxo-olean-12-ene-29-oic acid (5n): cream colored sticky solid; HPLC: t_R = 13.7 min (96% purity); yield: 97%; m.p. 260-262 °C; IR ($CHCl_3$): ν_{max} 3416, 2923, 2852, 1729, 1656, 1463, 1463, 1385, 1256, 1210, 1151, 1084, 1018 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 7.36-7.28 (m, 4H, Ar-4´,5´,6´,7´), 5.59 (s, 1H, CH-12), 5.10 (m, 2H, CH_2-1´), 3.23 (dd, *J* = 4.0, 8.0 Hz, 1H, CH-3), 2.78 (tt, 1H, CH-18), 2.33 (s, 1H, CH-9), 2.01 (dd, 2H, CH-2),1.65 (dd, 2H, CH-1), 1.35 (s, 3H, Me-27), 1.15 (s, 3H, Me-25), 1.14 (s, 3H, Me-29), 1.12 (s, 3H, Me-26), 0.98 (s, 3H, Me-23), 0.81 (s, 1H, Me-24), 0.75 (s, 3H, Me-28), 0.71 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 200.2 (C-11), 176.1 (C-30), 169.0 (C-13), 134.6 (C- 2´), 134.1 (C- 3´), 129.6 (C-7´), 128.8 (4´,5´,6´) ,128.5 (C-12), 78.7 (C-3), 65.4 (C-1´), 61.8 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 44.0 (C-20), 43.1 (C-8), 41.0 (C-19) , 39.1 (C-1, C-4), 37.6 (C-22), 37.0 (C-10), 32.7 (C-7), 31.7 (C-17), 31.1 (C-21), 28.4 (C-29), 28.2 (C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.3 (C-16), 23.3 (C-27), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 595.3549 $[M+H]^+$ calcd for $C_{37}H_{51}ClO_4 + H^+$ (595.3548).

3-(Benzo[1,3]dioxol-5-ylmethoxy)-11-oxo-olean-12-ene-29-oic acid (5o): colorless oil; HPLC: t_R = 12.6 min (96% purity); yield: 95%; IR ($CHCl_3$): ν_{max} 3391, 2924, 2876, 1723, 1649, 1443, 1490, 1386, 1249, 1211, 1154, 1095, 1018 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): *δ* (ppm) 6.87-6.77 (m, 3H, Ar-3´,4´,7´), 5.97 (s, 2H, CH-8´), 5.52 (s, H, CH-12), 5.10 (m, 2H, CH_2-1´), 3.23 (dd, *J* = 4.0, 12.0 Hz, 1H, CH-3), 2.80 (tt,1H, CH-18), 2.32 (s, 1H, CH-9),1.35 (s, 3H, Me-27), 1.14 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.11 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.81 (s, 1H, Me-24), 0.80 (s, 3H, Me-28), 0.75 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* (ppm) 200.1 (C-11), 176.2 (C-30), 169.1 (C-13), 147.8 (C-6´), 147.8 (C-5´), 129.9 (C-2´), 128.5 (C-11), 122.2 (C-3´), 109.0 (C-7´), 108.3 (C-4´), 101.2 (C-8´), 78.7 (C-3), 66.1 (C-1´), 61.7 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 44.9 (C-20), 43.1 (C-8), 41.0 (C-19), 39.1 (C-1, C-4), 37.6 (C-22), 37.0 (C-10), 32.7 (C-7), 31.7 (C-17), 31.1 (C-21), 28.4 (C-29), 28.2 (C-28), 28.1 (C-23), 27.2 (C-2), 26.4 (C-15), 26.4 (C-16), 23.3 (C-27), 18.6 (C-26), 17.4 (C-6), 16.4 (C-25), 15.6 (C-24); HR-ESIMS: *m/z* 605.3835 $[M+H]^+$ for calcd for $C_{38}H_{51}O_6$ + H^+ (605.3836).

3-(3-Bromo-4-methoxy-benzyloxy)-11-oxo-olean-12-ene-29-oic acid (5p): white solid; HPLC: t_R = 10.6 min (100% purity); yield: 90%; m.p. 300-301 °C; IR ($CHCl_3$): ν_{max} 3400, 2924, 2853, 1726, 1656, 1500, 1462, 1385, 1280, 1209, 1151, 1053, 1021 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): *δ* 7.55 (m, 1H, Ar-3´), 7.30 (m, 1H, CH-7´), 6.90 (m, 1H, CH-5´), 5.54 (s, 1H, CH-12), 5.09 (dd, 1H,CH_2-1´), 3.91 (s, 3H, OMe), 3.23 (dd, *J* = 4.0 Hz, 8.0 Hz 1H, CH-3), 2.78 (tt, 1H, CH-18), 2.32 (s, 1H, CH-9), 1.34 (s, 3H, Me-27), 1.14 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.11 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.80 (s, 1H, Me-24), 0.74 (s, 3H, Me-28), 0.68 (d, 1H, CH-5); ^{13}C NMR (100 MHz, $CDCl_3$): *δ* (ppm) 200.2 (C-11), 176.3 (C-30), 169.1 (C-13), 156.0 (C-5´), 133.6 (C-3´,2´), 128.9 (C-7´), 128.5 (C-12), 112.0 (C-6´),111.7 (C-4´), 78.9 (C-3), 65.2 (C-1´), 61.9 (C-9), 56.4 (C-5), 55.7 (-OCH_3), 48.3 (C-18), 45.4 (C-14), 44.0 (C-20), 43.3 (C-8), 41.2 (C-19), 39.2 (C-1, C-4), 37.7 (C-22), 37.2 (C-10), 32.8 (C-7), 31.9 (C-17), 31.2 (C-21), 28.6 (C-29), 28.3 (C-28), 28.2 (C-23), 27.4(C-2), 26.5 (C-15), 26.5 (C-16), 23.5 (C-27), 18.8 (C-26), 17.6 (C-6), 16.5 (C-25), 15.7 (C-24); HR-ESIMS: *m/z* 669.3114 and 671.3098 $[M+H]^+$ calcd for $C_{38}H_{53}BrO_5 + H^+$ (669.3149 and 671.3128).

3-(3,4-Dimethoxy-benzyloxy)-11-oxo-olean-12-ene-29-oic acid (5q): colorless oil; HPLC: t_R = 7.8 min (100 % purity); yield: 90%; IR ($CHCl_3$): ν_{max} 3434, 2930, 2867, 1724, 1656, 1517, 1463, 1385, 1263, 1210, 1159, 1084, 1024 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): *δ* 6.95-6.85 (m, 3H, Ar-3´,6´,7´), 5.57 (s, 1H, CH-12), 5.11 (dd, 1H, CH_2-1´), 3.89 (s, 6H, OMe), 3.22 (dd, *J* = 8.0, 12.0 Hz, 1H, CH-3), 2.77 (tt, 1H, CH-18), 2.32 (s, 1H, CH-9), 1.35 (s, 3H, Me-27), 1.14 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.10 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.80 (s, 1H, Me-24), 0.72 (s, 3H, Me-28), 0.67 (d, 1H, CH-5); ^{13}C NMR: (100 MHz, $CDCl_3$): *δ* (ppm) 200.1 (C-11), 176.3 (C-30), 169.2 (C-13), 149.0 (C-5´), 148.8 (C-4´), 128.7 (C-2´), 128.4 (C-12), 121.3 (C-7´), 111.6 (C-3´), 111.0 (C-6´), 78.9 (C-3), 66.2 (C-1´), 61.9 (C-9), 55.9 (C-5), 55.5 (OMe), 54.9 (OMe), 48.2 (CH-18), 45.3 (C-14), 43.9 (C-20), 43.1 (C-8), 41.0 (C-19), 39.1 (C-1, C-4), 37.6 (C-22), 37.0 (C-10), 32.7 (C-7), 31.7 (C-17), 31.1 (C-21), 28.4 (C-29), 28.2 (C-28), 28.1 (C-23), 27.2(C-2), 26.4(C-15), 26.3 (C-16), 23.3 (C-27), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.6 (C-24); HR-ESIMS: *m/z* 621.4145 $[M+H]^+$ for calcd for $C_{39}H_{56}O_6$ + H^+ (621.4149).

3-(2,4-Dichloro-benzyloxy)-11-oxo-olean-12-ene-29-oic acid (5r): yellow oil; HPLC: t_R = 7.3 min (100% purity); yield: 92%; IR ($CHCl_3$): ν_{max} 3454, 2927, 2866, 1730, 1654, 1590, 1464, 1386, 1365, 1326, 1312, 1279, 1102, 1084, 1048 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): *δ* (ppm) 7.43-7.27 (m, 3H, Ar-4´,6´,7´), 5.60 (s, 1H, CH-12), 5.30-5.19 (dd, 2H, CH_2-1´), 3.23 (dd, *J* = 4.0 Hz, 8.0 Hz 1H, CH-3), 2.80 (tt, 1H, CH-18), 2.33 (s, 1H, CH-9), 1.36 (s, 3H, Me-27), 1.15 (s, 3H, Me-25), 1.13 (s, 3H, Me-29), 1.12 (s, 3H, Me-26), 1.00 (s, 3H, Me-23), 0.81 (s, 1H, Me-24), 0.80 (s, 3H, Me-28), 0.77 (d, 1H, CH-5);^{13}C NMR (100 MHz,

$CDCl_3$): δ (ppm) 200.1 (C-11), 176.0 (C-30), 168.9 (C-13), 134.8 (C-5´), 134.5 (CH-3´), 132.3 (C-2´) , 130.9 (C-7´), 129.5 (C-4´), 128.6 (C-12), 127.2 (C-6´), 78.7 (C-3), 63.8 (C-1´) ,61.8 (C-9), 54.9 (C-5), 48.2 (C-18), 45.3 (C-14), 44.1 (C-20), 43.2 (C-8), 41.0 (C-19), 39.1 (C-1, C-4), 37.7 (C-22), 37.0 (C-10), 32.7 (C-7), 31.8 (C-17), 31.1 (C-21), 28.4 (C-29), 28.3 (C-28), 28.1 (C-23), 27.3 (C-2), 26.4 (C-15), 26.4 (C-16), 23.3 (C-27), 18.6 (C-26), 17.4 (C-6), 16.3 (C-25), 15.5 (C-24); HR-ESIMS: *m/z* 629.3164 $[M+H]^+$ calcd for $C_{37}H_{50}Cl_2O_4 + H^+$ (629.3158).

Cell culture and growth conditions: PC-3 prostate cancer, MIAPaCa-2 pancreatic cancer, and HepG2 hepatocellular carcinoma cells were originated from EACC, UK (European Collection of Cell Culture) and were purchased through Sigma-Aldrich India. The cells were grown in RPMI- 1640 or MEM medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), streptomycin (100 µg/mL), L-glutamine (0.3 mg/mL), pyruvic acid (0.11 mg/mL), and 0.37% $NaHCO_3$. Cells were grown in a CO_2 incubator (Thermocon Electron Corporation, MA, USA) at 37 ℃ in an atmosphere of 95% air and 5% CO_2 with 98% humidity. Camptothecin and paclitaxel were used as a positive controls in this study.

Cell proliferation assay: MTT assay was performed to determine the cell viability. Cells were seeded in 96 well plates and exposed to different concentrations of synthesize molecules for 48 h. MTT dye (2.5 mg/ml in PBS) was added 4 hours priors to experiment termination. The plates were then centrifuged at 1500 RPM for 15 min and the supernatant was discarded, while the MTT formazan crystals were dissolved in 150 µL of DMSO. The OD measured at 570 nm with reference wavelength of 620 nm.[21]

DNA content and cell cycle phase distribution: PC-3 cells were treated with 5, 10 and 20 µM concentrations of compound **5b** for 24 h. Cells were collected, washed in PBS, fixed at 70% cold ethanol and placed at −20 °C overnight. Cells were again washed with PBS, subjected to RNase digestion (400 µg/ml) at 37 °C for 45 min. Finally, cells were incubated with propidium iodide (10 µg/ml) for 30 min and analyzed immediately on a flow cytometer FACSCalibur (Becton Dickinson, USA). The data were collected in list mode on 10,000 events and illustrated in a histogram, where the number of cells (counts) is plotted against the relative fluorescence intensity of PI (FL-2; λem: 585 nm; red fluorescence). Resulting DNA distributions were analyzed by Modfit (Verity Software House Inc., Topsham, ME) for the proportions of cells in apoptosis, G_1-phase, S- phase, and G_2-M phases of the cell cycle.[21]

Fluorescence microscopy: PC-3 cells were treated with 5, 10 and 20 µM concentrations of compound **5b** for 24 h. After treatment cells were collected, washed with PBS twice and fixed in 400 µl cold acetic acid: methanol (1+3, v/v) overnight at 4 °C. Next day cells washed with fixing solution and dispensed in 50 µl of fixing solution. Spread cells on a clean slide and dried overnight at room temperature. Cells were stained with Hoechst 33258 (5 µg/ml in 0.01 M citric acid and 0.45 M disodium phosphate containing 0.05% Tween 20) for 30 min at room temperature. After 30 min slides were washed with distilled water followed by in PBS. While wet, 40 µl of mounting fluid (PBS: glycerol, 1/1) was poured over the slide and covered with a glass cover slip and sealed with nail polished. Cells were observed under microscope for any nuclear morphological changes occur in apoptosis.[21]

Flow cytometric determination of mitochondrial membrane potential: Changes in mitochondrial transmembrane potential ($\Delta\psi_m$) as a result of mitochondrial perturbation were measured after staining with Rhodamine-123.[21] PC-3 cells were incubated with the indicated doses of compound **5b** for 24 h. Rhodamine-123 (5 µM) was added 1 h before the termination of the experiment and cells were collected, washed in PBS. The fluorescence intensity of 10,000 events was analyzed in FL-1 channel on a BD FACS Calibur (Becton Dickinson, USA) flow cytometer. The decrease in fluorescence intensity caused by loss of mitochondrial membrane potential was analyzed in FL-1 channel.

Western blot analysis: Cells were treated with different concentration of compound **5b** for 24 h. Cells were collected at 400×*g* at 4 °C, washed in PBS twice and cell pellets were incubated with cold RIPA buffer (Sigma Aldrich, India)) containing 50 mM NaF, 0.5 mM $NaVO_4$, 2 mM PMSF and 1% protease inhibitor cocktail for 40 min. Cells were centrifuged at 12000 x g for10 min at 4 °C and the supernatant was collected as whole cell lysates for western blot analysis of various proteins. Protein was measured employing Bio-Rad protein assay kit using bovine serum albumin as standard. Proteins aliquots (30-70 µg) were resolved on SDS-PAGE and then electro transferred to PVDF membrane overnight at 4 °C at 30V. Nonspecific binding was blocked by incubation with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The blots were probed with respective primary antibodies (purchased from Santacruz Biotech) for 2 h and washed three times with TBST. The blots were then incubated with horseradish peroxidase conjugated mouse or rabbit secondary antibodies (purchased from Santacruz Biotech.) for 1 h, washed again three times with TBST and signals detected using ECL plus chemiluminescence's kit on X-ray film.[21]

Wound healing migration assay*:* The wound-healing migration assay was performed as described previously.[23] Briefly, PC-3 cells were treated with mitomycin-C to inactivate cell proliferation, wounded by micro tip, washed with PBS, supplemented with fresh medium treated with SAD. Images of the cells were taken after ~0 to 24 h of incubation and the percentage of wound closure was expressed with respect to untreated cells consider 100%. The wound-healing migration assay in HUVEC cells was also done using similar protocol.

Statistical analysis. Data expressed as mean ± SD or representative of one of three similar experiments unless otherwise indicated. Comparisons were made between control and treated groups or the entire intra group using one way ANOVA with post Bonferroni test through GraphPad Prism

5.00.288 statistical analysis software. *p* -values *<0.001 were considered significant.

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