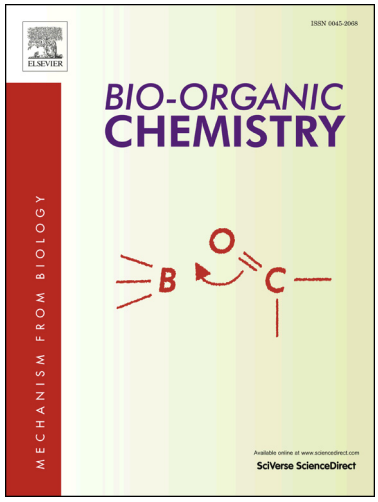

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Revised

Synthesis of ring-C modified oleanolic acid derivatives and their cytotoxic evaluation

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ABSTRACT

Ring-C of oleanolic acid was chemically modified by treating with NBS under a variety of experimental conditions. The structures of the synthesized compounds were established by spectral analysis (^{1}H &^{13}C NMR and Mass). All the compounds were evaluated against a panel of five human cancer cell lines by using MTT assay. Among the tested compounds, **2** and **7** showed significant activity against breast cancer cell line, MCF-7. Most significantly, compound **7** showed several folds enhanced activity against MCF-7 cancer cell lines (IC_{50}:2.96 µM) than that of the parent (**1**) and the intermediate compound (**6**). Flow cytometric analysis revealed that these compounds arrested the cell cycle in G0/G1 phase and induced mitochondrial mediated apoptosis.

Keywords: Oleanolic acid, N-bromosuccinimide, Anticancer activity, Apoptosis, Caspase 9 activity

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1. Introduction

Oleanolic acid (3β-Hydroxy-olea-12-en-28-oic acid), a natural pentacyclic triterpenoid (PCT) widely exists in plants in free or glycosidic forms [1]. It attracted the attention of researchers, due to its broad spectrum bioactivity and it plays a key role in human health care with high efficacy and low toxicity [2]. Literature search reveals that, it has been isolated from more than 1620 plant species including many food and medicinal plants [3]. The hepatoprotective effects of oleanolic acid allow its use in China as an over-the-counter oral drug to treat liver disorders [4]. In addition to this, oleanolic acid has also been found to exhibit highly potent anti-inflammatory, antitumor, anti-HIV, cardiovascular and glycogen phosphorylase inhibitory activities [5,6]. Recently, oleanolic acid was patented in China as an injectable for hepatitis B and liver cancer [7]. In view of the above pharmacological properties, oleanolic acid proves to be a good starter molecule for further synthetic modifications [8,9]. Oleanolic acid has three ‘‘active’’ sites, i.e. the C-3 hydroxyl,C12-C13 double bond in ring-C and C-28 carboxylic acid, which are amenable for chemical modifications to make a series of new chemical entities [10,11]. The ring-C has an advantage with a double bond present between C12-C13, leaving the allylic C-11 position for substitution reactions such as halogenation. The resultant C-11 halo substituted intermediates can be transformed further into a range of products such as dienes, α,β-unsaturated ketones, saturated and unsaturated lactones. It has been reported in literature that modification of ring-C enhances the biological activities, such as anti-inflammatory, anti-diabetic, nephropathy and cytotoxicity [12,13]. Further, Siewert *et al.* reported some 12-hydroxy or halo 28→13 lactones by modifying ring-C of oleanane type triterpenes with significantly enhanced *in vitro* cytotoxicity [14]. Recently, we have reported the synthesis of some

ring-C modified analogues of ursolic acid, the regioisomer of oleanolic acid with enhanced cytotoxicity [15]. With this background and in continuation of our discovery programme to identify highly potent and selective antitumor agents based on pentacyclic triterpenic acids, a series of ring-C modified frameworks of oleanolic acid were synthesized and evaluated for their cytotoxic potential against A-549 (Colon), DU-145 (Prostate), MCF-7 (Breast), HeLa (Cervical) and ACHN (Renal) human cancer cell lines. The potent molecules were subjected to apoptosis and other cytometric studies.

2. Results and discussions

2.1. *Chemistry*

The protocols employed for the synthesis of ring-C analogues of oleanolic acid are presented in Scheme 1. Oleanolic acid (**1**) when treated with N-bromosuccinimide in CCl_4 at room temperature afforded 3β-Hydroxy-olean-28→13-olide [**2,** IR (1760 cm^{-1}) and ^{13}C NMR (δ 178.78 ppm)] in 45 % yield. The same reaction was then extended to oleanolic acid protected at C-3 hydroxyl or C-17 carboxylic acid or both in CCl_4 or dioxane-$CaCO_3$ solvent systems. The C-3 hydroxyl protected oleanolic acid acetate (**3**) on treatment with NBS yielded an unsaturated lactone **4** [IR: 1758 (γ-lactone), 1720 & 1240 (C-3-acetate); ^{13}C NMR: δ 178.15, 156.96, 121.76 ppm] in CCl_4 and a saturated lactone **5** [IR: 1760 cm^{-1}; ^{13}C NMR:δ 178.87 (γ-lactone)] in aq. dioxane-$CaCO_3$ each in 47% yield. Interestingly, the C-17 carboxylic acid protected oleanolic acid methyl ester (**6**) under identical experimental conditions yielded the same methyl-3β–hydroxy-olean-9(11),12-dien-28-oate [**7**,^{13}C NMR at δ 170.96 (C=O), 115.78 (C12), 120.44 (C9), 145.14 (C11), 154.16 (C13)] in both the cases with 32% and 30% yields respectively. In

another set of reaction, both the C-17 carboxylic acid and C-3 hydroxyl protected 3-acetoxy-17-carbomethoxy-oleanolic acid (**8**) was subjected to similar NBS reactions. In aq. dioxane, it afforded methyl-3β–acetoxy-olean-11-oxo-12-ene-28-oate **9** [IR: 1720 cm^{-1}; ^{13}C NMR: δ 200.15 (C11), 177.49 (C28) ppm] in 50% yield. Surprisingly, it did not yield any product in CCl_4, but only underwent decomposition.

2.2 Biology

2.2.1 *In vitro cytotoxicity*

The synthesized compounds (**2-9**) along with the parent (**1**) were evaluated for their *in vitro* anticancer activity against a panel of five human cancer cell lines by employing MTT assay using doxorubicin as the standard [16]. The results were summarized in Table 1. The cytotoxicity values were expressed as IC_{50} in μM concentration. The *in vitro* screening results revealed that, most of the tested compounds showed potent anticancer activity with IC_{50} values ranging from 2.10±0.65 to 9.42±1.47μM. From the IC_{50} values, we concluded that:

a) Among the tested compounds, **2**, **4, 5** and **7** showed highly potent anticancer activities against all the cancer cell lines used.

b) Compound **7** was found to be the most active one exhibiting highest activity against four cell lines namely MCF-7 (IC_{50}:2.96 μM), HeLa (IC_{50}:6.30 μM), DU-145 (IC_{50}:6.77 μM) and ACHN (IC_{50}:5.58 μM). The anticancer activity of this compound against MCF-7 cell line is 10.5 fold higher than intermediate compound **6** and several folds higher than the parent compound (**1**). Close evaluation of the IC_{50} values of the compound (**7**) suggests that, introduction of a conjugated double bond system in ring-C of the parent acid increases the *in vitro* cytotoxicity potency to several folds.

c) Compound **2** showed highest activity against A-549 cell line (IC_{50}:2.10 μM) followed by MCF-7 (IC_{50}:4.6 μM). In fact, this compound exhibited maximum activity against A-549 among the tested compounds with several fold enhancement over the parent compound and more than the standard doxorubicin (IC_{50}:2.17 μM).

d) Among the lactones **2** and **5**, the lactone (**2**) with a C-3 hydroxyl group showed higher activity against MCF-7 & A-549, whereas lactone **5** with C-3 acetoxy functionality found to exhibit higher activity against Hela, DU-145 and ACHN.

e) Similarly, between the lactones **4** and **5**, the unsaturated lactone (**4**) found to be more active against MCF-7, A-549, DU-145, ACHN, whereas the saturated one (**5**) is more active against Hela cell line.

As we have been working on identifying the potent leads for breast cancer cell lines [17], compound **7** with highest activity has been taken up for further biological evaluation.

2.2.2 *Cell cycle analysis*

Many anticancer compounds exert their growth inhibitory effect either by arresting the cell cycle at a particular checkpoint or by induction of apoptosis or a combined effect of both cell cycle arrest and apoptosis [18,19]. Furthermore, regulation of the cell cycle and apoptosis are considered to be effective cancer therapeutic methods [20]. *In vitro* screening results revealed that compound **7** showed significant anticancer activity against human breast cancer cell line, MCF-7. Therefore, it was considered of interest whether the inhibition of cell growth was on account of cell cycle arrest. In this study MCF-7 cells were treated with compound (**7**) at 2.5 and 5 μM concentrations along with compound **6** at 5 μM concentration for 48h. The data obtained

from the cytometric profile clearly indicated that, compound **7** arrested cell cycle at G0/G1 phase (86.99%, 92.15%) compared to the untreated control (84.46%) and the intermediate **6** (86.21%) (Figure 1).The population of G2 phase decreased (from 6.92% to 3.37%) with increase in concentration from 2.5 μM to 5.0 μM for compound **7**, whereas for compound **6** at 5 μM concentration it was (8.44%) compared to control (8.59%).

2.2.3 *Measurement of mitochondrial membrane potential (ΔΨm)*

The maintenance of mitochondrial membrane potential ((ΔΨm) is significant for mitochondrial integrity and bio energetic function [21]. Mitochondrial changes, including loss of mitochondrial membrane potential (ΔΨm), are key events that take place during drug-induced apoptosis. Mitochondrial injury by **6** and **7** was evaluated by detecting fall in mitochondrial membrane potential (ΔΨm). In this study we have investigated the involvement of mitochondria in the induction of apoptosis by **6** and **7**. After 48h of drug treatment with these compounds, it reduced the mitochondrial membrane potential (ΔΨm) of MCF-7 cells, as observed by JC-1 staining (Figure2).

2.2.4 *Effect on intracellular ROS generation*

Many anticancer agents have demonstrated to exert their cytotoxic effects by the generation of reactive oxygen species (ROS) [22,23], which is considered as one of the key mediators of apoptotic signaling. Therefore, we decided to investigate the role of these compounds **6** and **7** in inducing the production of ROS that could potentially lead to the cytotoxic effect in the MCF-7 cells. In order to demonstrate the role of test compounds on ROS generation during apoptosis process, production of ROS was

examined by using an oxidant-sensitive fluorescent probe, DCFDA (2', 7'dichlorofluorescin diacetate). After treatment with these compounds, the level of ROS was significantly increased (Figure 3). The ratio of DCF-positive cells for compound **6** is 7.6 % at 5 µM and for compound **7** are 7.3 and 12.0 % at 2.5 and 5 µM respectively. The test results evidenced that these compounds have enhanced the generation of ROS in MCF-7 cells.

2.2.5 *Effect on caspase-9*

The activation of caspases plays an important role in the process of programmed cell death or apoptosis. Caspases, or cysteine aspartic protease, are a family of cysteine proteases, which are crucial mediators of apoptosis. The MCF-7 cells lack endogenous caspase-3, whereas caspase-9 plays an important role in mediating drug-induced apoptosis [24]. MCF-7 cells were treated with these compounds (**6** and **7**) for 48h. The results demonstrated that there was 2-3 fold induction in caspase-9 activity when compared to untreated control (Figure 4), which suggests that they have the ability to induce cell death by apoptosis in MCF-7 cells.

3. Conclusion

The chemical transformation of ring C of oleanolic acid was resulted in the synthesis of some modified frameworks. The cytotoxicity evaluation of these compounds revealed that the diene functionality in ring C (**7**) drastically enhances the activity against four of the tested cell lines and most effective towards the MCF-7, breast cancer cell line. Flow cytometric analysis revealed that this compound arrested the cell cycle at G0/G1 phase and induced mitochondrial mediated apoptosis. Therefore,

compound **7** can be considered as lead molecule for further development against breast cancer.

4. Experimental

4.1. *Chemistry*

Melting points were determined in open capillaries on a Buchi melting point apparatus and are uncorrected. IR spectra were recorded in KBr disks on JASCO FTIR 5300 spectrophotometer and wave numbers are expressed in cm^{-1}. ^{1}Hand ^{13}C NMR spectra were recorded on Bruker500 MHz spectrometers and chemical shifts were reported as part per million (ppm) with tetramethylsilane as an internal standard. Mass spectra were obtained on Waters Synapt mass instrument. Column chromatography was performed on ACME grade 60-120 mesh silica gel and Merck pre-coated silica gel 60 F254 plates were used for thin layer chromatography.

4.1.1. *Oleanolic acid **1***

Oleanolic acid was isolated from the leaves of *Diospyros melanoxylon* in 0.1% yield, by our reported procedure [25].

4.2.2 *General procedure for NBS reaction in CCl_4*

To a stirred solution of **1**, **3**, **6** and **8**(0.4 mmol) in CCl_4 (5ml), freshly recrystallized NBS (0.6 mmol) were added at room temperature. After continuous stirring for 8h, the reaction mixture was filtered and concentrated under reduced pressure to yield the crude product and chromatographed over silica gel column to afford compound **4**.

4.2.3 *3β-Hydroxy-olean-28→13-olide(**2**)*

Colorless solid (45 %); mp: 235-238 ^{0}C; IR (KBr) ν max: 3400, 2950, 1760, 1710, 1460, 1380 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$, ppm):δ 0.78 (3H, s), 0.89(3H, s), 0.91(3H, s), 1.00 (3H, s), 1.22 (3H, s), 1.44 (3H, s), 1.99 (3H, s), 2.34(1H, m) 3.26(1H, m) ;^{13}C NMR (125 MHz, $CDCl_3$): δ 15.38 (C_{25}), 16.91(C_{27}), 17.70 (C_6), 19.07 (C_{11}), 21.09 (C_{26}), 21.34 (C_{16}), 23.55 (C_{24}), 27.18 (C_{23}), 27.55 (C_{22}), 27.97 (C_{15}), 29.18 (C_2), 30.46 (C_{20}), 30.87 (C_{30}), 31.86 (C_{29}), 33.25 (C_{12}), 33.89 (C_{21}), 34.64 (C_7), 36.57 (C_{10}), 38.35 (C_{19}), 38.90 (C_1), 39.95 (C_8), 42.45 (C_4), 43.42 (C_{14}), 45.58 (C_{18}), 52.35 (C_{17}), 55.23 (C_9), 56.48 (C_5), 78.72 (C_3), 91.61 (C_{13}, $\underline{C}H_2$), 178.78 (C_{28}, $\underline{C}O$);ESI-HRMS (m/z) $[M - H]^+$ calcd for $C_{30}H_{47}O_3$ 455.3525, found 455.3543.

4.2.4 *3β–Acetoxy-olean-11-ene-28→13-olide (**4**)* [26]

Colorless solid (0.046 g, 46.9%); mp: 221-225 ^{0}C; IR(KBr) ν max: 2950, 1900, 1758, 1720, 1460, 1380, 1240 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88, 0.91, 0.94, 0.97, 1.01, 1.18, 2.06 , 2.95 (1H m), 4.52 (1H, m), 5.55 (d, *J* = 6.04 Hz, 1H), 5.58 (d, *J* = 6.04 Hz, 1H); ^{13}C NMR(125 MHz, $CDCl_3$): δ 14.95 (C_{27}), 19.86 (C_6), 20.61 (C_{25}), 21.29 (C_{26}), 25.64 (C_2), 26.03 (C_{16} &$\underline{C}H_3CO$), 27.11 (C_{15}), 29.16 (C_{23}), 29.70(C_{24}), 30.83 (C_{11}), 31.80 (C_{30}), 32.14 (C_{29}), 32.20 (C_7), 33.83 (C_{22}), 33.89 (C_{20}), 37.03 (C_{21}), 37.46 (C_{19}), 38.34 (C_{10}), 39.47 (C_4), 41.43 (C_1), 43.58 (C_8), 47.49 (C_{14}), 50.73 (C_{17}), 51.09 (C_{18}), 51.62 (C_9), 55.51 (C_5), 65.38 (C_3 & C_{13}), 121.76 (C_{11} & C_{12}), 156.96 ($CH_3\underline{C}O$), 178.15 (C_{28}, $\underline{C}O$); ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{32}H_{49}O_4$ 497.3631, found 497.3633.

4.2.5 *General procedure for NBS reaction in aq dioxane-$CaCO_3$*

To a stirred solution of **1**, **3**, **6** and **8**(0.4 mmol) in dioxane (5ml), freshly recrystallized NBS (0.6mmol)were added at room temperature with water (0.5 ml)

followed by $CaCO_3$(0.4 mmol). After continuous magnetic stirring for 18h, the reaction mixture was filtered. The resultant filtrate was diluted with ice cold water (5 ml) and extracted with ethyl acetate (3 x5 ml). The combined organic layer was dried over anhydrous sodium sulphate and concentrated under reduced pressure to yield the crude product. Then the crude product was chromatographed over silica gel column to afford compound **5, 7** and **9**.

4.2.6 *3β–Acetoxy-olean-28→13-olide(**5**)* [27]

Colorless amorphous solid (0.546 g, 92%); mp: 178-180 ^{0}C; IR (KBr) ν max: 2950, 1900, 1760, 1450, 1380, 1210 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.86, 0.88, 0.90, 0.92, 0.97, 1.00, 1.22, 1.44, 1.99, 2.05, 2.35 (1H, m), 4.55 (1H, m); ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.49 (C_{26}), 17.03 (C_{24}), 17.59 (C_{25}), 19.10 (C_6), 21.13 (C_{27}), 21.32 (C_{12}), 23.45 ($\underline{C}H_3CO$), 23.57 (C_{16}), 27.54 (C_{30}), 27.88 (C_{11}), 29.18 (C_{15}), 29.70 (C_{23}), 30.45 (C_2), 30.92 (C_{20}), 31.87 (C_{22}), 33.26 (C_7), 33.87 (C_{29}), 34.58 (C_{21}), 36.47 (C_{10}), 37.83 (C_4), 38.04 (C_1), 39.93 (C_8), 42.45 (C_{19}), 43.41 (C_{14}), 45.53 (C_{18}), 52.33 (C_{17}), 55.32 (C_9), 56.22 (C_5), 80.59 (C_3), 91.65 (C_{13}), 170.97 ($CH_3\underline{C}O$), 178.87 (C_{28}, $\underline{C}O$); ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{32}H_{50}O_4$ 499.3787, found 499.3767.

4.2.7 *Methyl-3β–hydroxy-olean-9(11), 12-dien-28-oate(**7**)* [28]

Colourless amorphous solid (0.546 g, 92%); mp: 138-140 ^{0}C; IR (KBr) ν max: 3460-3380, 1720, 1450, 1360, 1260, 1220 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.83, 0.87, 0.92, 0.98, 1.00, 1.02, 1.18, 3.04 (br. dd, *J* = 17.37, 3.77 Hz, 1H) , 3.64 (s, 3H, $OC\underline{H}_3$), 5.63 (d, *J* = 6 Hz, 1H), 5.65 (d, *J* = 6 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 16.72 (C_6), 18.10 (C_{25}), 20.09 (C_{26}), 21.29 (C_{15}), 23.67 (C_{24}), 24.16 (C_{23}), 25.04 (C_{16}), 25.10 (C_{27}), 26.86 (C_{30}), 28.09 (C_{29}), 30.64 (C_2), 32.05 (C_{20}), 32.13 (C_{22}), 32.92 (C_7),

33.67 (C_{21}), 36.70 (C_1), 37.82 (C_4), 38.63 (C_{10}), 39.61 (C_8), 40.63 (C_{18}), 42.26 (C_{14}), 45.75 (C_{19}), 46.05 (C_{17}), 51.18 ($O\underline{C}H_3$), 51.64 (C_5), 80.51 (C_3), 115.78 (C_{11}), 120.44 (C_{12}), 145.14 (C_9), 154.16 (C_{13}), 178.13 ($C_{28,}$ $\underline{C}O$); ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{31}H_{49}O_3$469.3682, found 469.3673.

4.2.8 *Methyl-3β–acetoxy-olean-11-oxo-12-ene-28-oate (**9**)* [29]

Colorless amorphous solid (0.546 g, 50%); mp: 217-220 ^{0}C; IR (KBr) ν max: 2960, 2925, 2850, 1720, 1460, 1360, 1240 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.87, 0.91, 0.93, 1.13, 1.35, 1.55-1.75 (m 9H), 2.05 (m, 1H), 2.34 (s, 3H), 2.81-2.84 (m, 1H), 2.98 (d, *J* = 3.77 Hz, 1H), 3.03 (d, *J* = 3.77 Hz, 1H), 3.64 (s, 1H), 5.56 (s, 1H);^{13}C NMR (125 MHz, $CDCl_3$): δ 16.24 (C_{24}), 16.68 (C_{25}), 17.33 (C_6), 18.96 (C_{26}), 21.29 (C_{16}), 22.98 (C_{30}), 23.45 ($\underline{C}H_3CO$), 23.53 (C_{27}), 23.59 (C_2), 27.77 (C_{15}), 28.09 (C_{23}), 29.69 (C_{20}), 30.68 (C_{22}), 31.63 (C_{29}), 32.86 (C_7), 33.74 (C_{21}), 37.17 (C_{10}), 38.07 (C_4), 38.81 (C_1), 41.63 (C_{18}), 43.50 (C_{14}), 44.27 (C_{19}), 45.07 (C_8), 46.25 (C_{17}), 51.87 (C_{31}), 55.13 (C_5), 61.72 (C_9), 80.65 (C_3), 127.89 (C_{12}), 168.63 (C_{13}), 177.49 ($C_{28,}$ & $C_{32,}$ $\underline{C}O$), 200.15 ($C_{11,}$ $\underline{C}O$) ; ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{33}H_{51}O_5$ 527.3736, found 527.3734.

4.3 Anticancer activity

The anticancer activity of the compounds was determined using MTT assay. Cells(1×10^4) per well were seeded in 100µl DMEM, supplemented with 10% FBS in each well of 96-well micro culture plates and incubated for 24h at 37°C in a CO_2 incubator. Compounds, diluted to the desired concentrations in culture medium, were added to the wells with respective vehicle control. After 48h of incubation, a volume of 10 µl MTT [3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide] (5 mg/mL) was added to each well and the plates were further incubated for 4h. The supernatant

from each well was carefully removed, formazon crystals were dissolved in 100µl of DMSO and absorbance at 540 nm wavelength was recorded.

4.3.1 *Cell cycle analysis*

Flow cytometric analysis (FACS) was performed to evaluate the distribution of the cells through the cell cycle phases. MCF-7, Breast cancer cells were incubated with compounds **6** and **7** for 48h. Untreated and treated cells were harvested, washed with PBS, fixed in ice-cold 70% ethanol and stained with propidium iodide (Sigma Aldrich). Cell cycle was performed by flow cytometry (Becton Dickinson FACS Caliber) as earlier described [30].

4.3.2 *Mitochondrial membrane potential*

MCF-7 (1×10^6 cells/well) cells were cultured in six-well plates after treatment with compounds **6** and **7** for 48h. Cells were collected by trypsinization after 48h of treatment and washed with PBS followed by resuspending in JC-1 (5 µg/ml) and incubated at 37^0C for 15 min. Cells were rinsed three times with medium and suspended in pre warmed medium. The cells were then subjected to flow cytometric analysis on a flow cytometer (Becton Dickinson) in the FL1, FL2 channel to detect mitochondrial potential [31].

4.3.3. *ROS generation*

The production of ROS (reactive oxygen species) was measured by flow cytometry using 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as previously described [32]. In this study MCF-7 cells were treated with compound **6** and **7** for 48h. After treatment, cells were incubated with DCF-DA at 37 ^{0}C for 30 min and then measured with the flow cytometer (FACS).

4.3.4 *Activation of Caspase 9*

To determine the caspase-9 activity of compounds **6** and **7** for detection of apoptosis in breast cancer cell line (MCF-7), the commercially available apoptosis detection kit (Caspase 9 Assay kit, Sigma Aldrich, St Louis, USA) was used. MCF-7 cells were treated with compounds **6** and **7** for 48h. After 48h of drug treatment assay was performed according to manufactures introduction and readings were taken at excitation wavelength 400 nm and emission wavelength 505 nm.

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Captions

Scheme 1: Synthesis of oleanolic acid ring C modified frameworks.

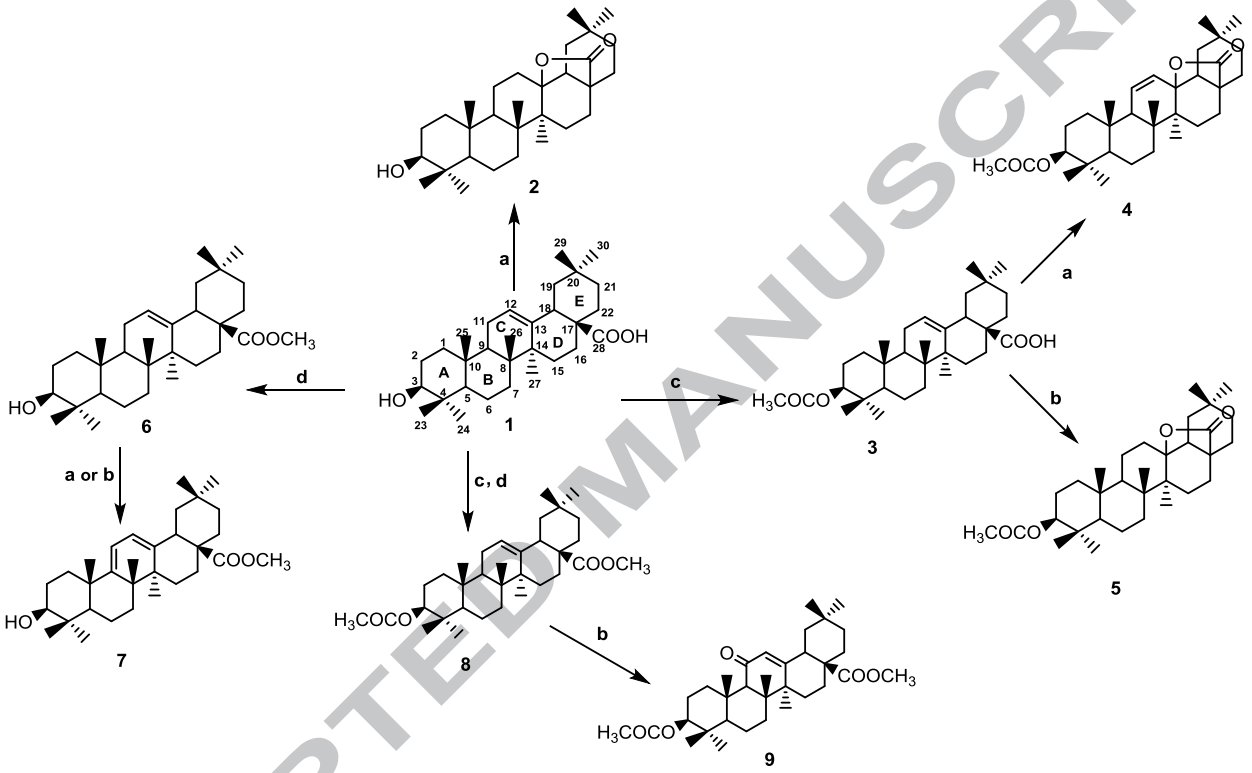
Table 1: *In vitro* anticancer activity of compounds **1-9.**

Figure 1: Cell cycle analysis of compounds **6** and **7** on MCF-7 cells.

Figure 2: Mitochondrial membrane potential of compound **6** and **7** in MCF-7 cells.

Figure 3: The effect of **6** and **7** on the ROS production in MCF-7 cells.

Figure 4: Effect of compounds **6** and **7** on caspase-9 activity.

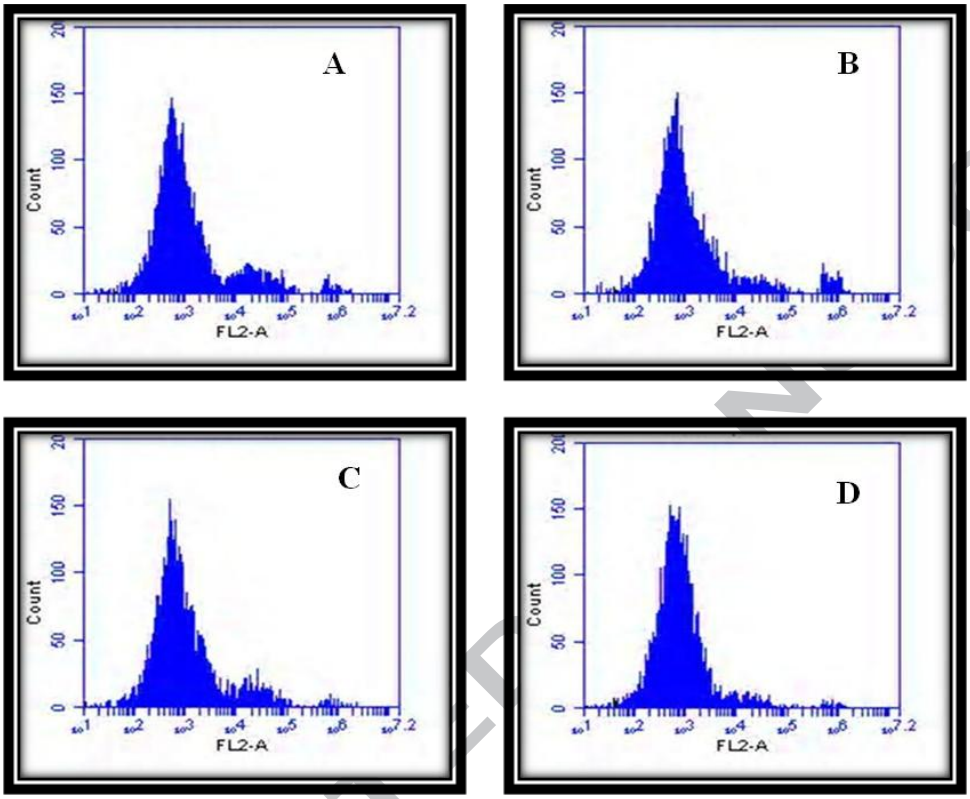


Scheme 1.Reagents and conditions a. NBS-CCl_4, rt, 8h b. NBS-aq.Dioxane-$CaCO_3$, rt 18h c. $(CH_3CO)_2O$-Py, rt, 12h, 98% d. CH_2N_2, Diethyl ether, 12h, 96%.

Table 1. IC_{50} values (in μM) of compounds in selected human cancer cell lines.

Compounds	MCF-7	A-549	Hela	DU-145	ACHN
1	> 100	> 100	> 100	53.49±2.04	> 100
2	4.6±0.20	2.10±0.65	30.96±0.75	33.05±0.36	30.10±0.98
3	> 100	> 100	> 100	> 100	> 100
4	9.42±1.47	15.30±2.83	13.41±0.29	12.38±2.19	10.63±0.35
5	15.05±1.33	17.00±2.69	7.04 ±0.49	28.31±1.73	27.71±1.26
6	31.29±0.04	48.88±1.62	36.49±0.052	51.45±1.02	55.80±2.83
7	2.96±0.15	19.60±1.80	6.30±0.13	6.77±2.57	5.85±0.32
8	> 100	> 100	> 100	> 100	> 100
9	16.68±0.45	44.44±0.22	32.86±0.61	24.06±1.61	> 100
Doxorubicin	0.90±0.37	2.17±0.54	5.05±0.48	1.83±0.75	2.74±0.36

Values represent mean ± SD, n=3



Sample	SubG1%	G0/G1%	S%	G2/M%
Control (MCF-7)	0.76	84.46	4.56	8.59
6 (5 µM)	0.79	86.21	2.61	8.44
7 (2.5 µM)	0.69	86.99	4.19	6.92
7 (5 µM)	0.62	92.15	2.10	3.37

Figure 1: Cell cycle analysis of compounds **6** and **7** on MCF-7 cells; A: Untreated control cells (MCF-7), B: **6** (5 µM), C: **7** (2.5 µM) and D: **7** (5 µM).

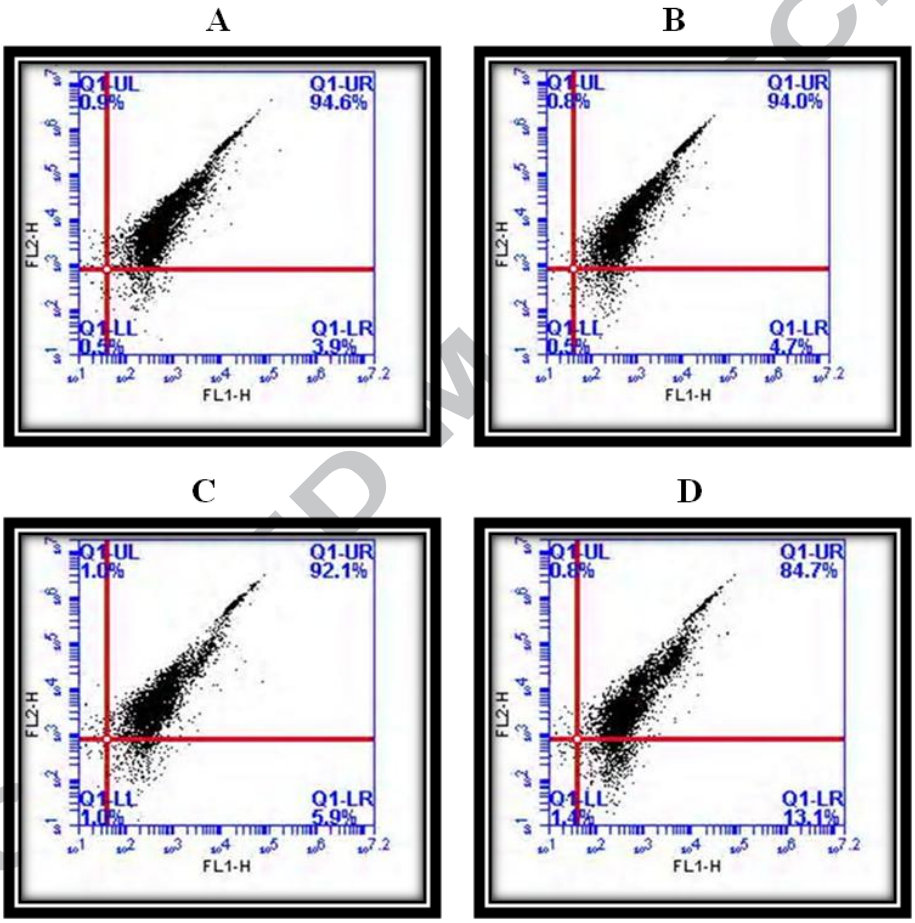


Figure 2: Drops in mitochondrial membrane potential (ΔΨm) was assessed by JC-1 staining of MCF-7 cells treated with compound **6** and **7** and samples were then subjected to flow cytometry analysis on a FACScan (Becton Dickinson); A: Untreated control cells (MCF-7), B:**6** (5 µM), C: **7** (2.5 µM) and D: **7** (5 µM).

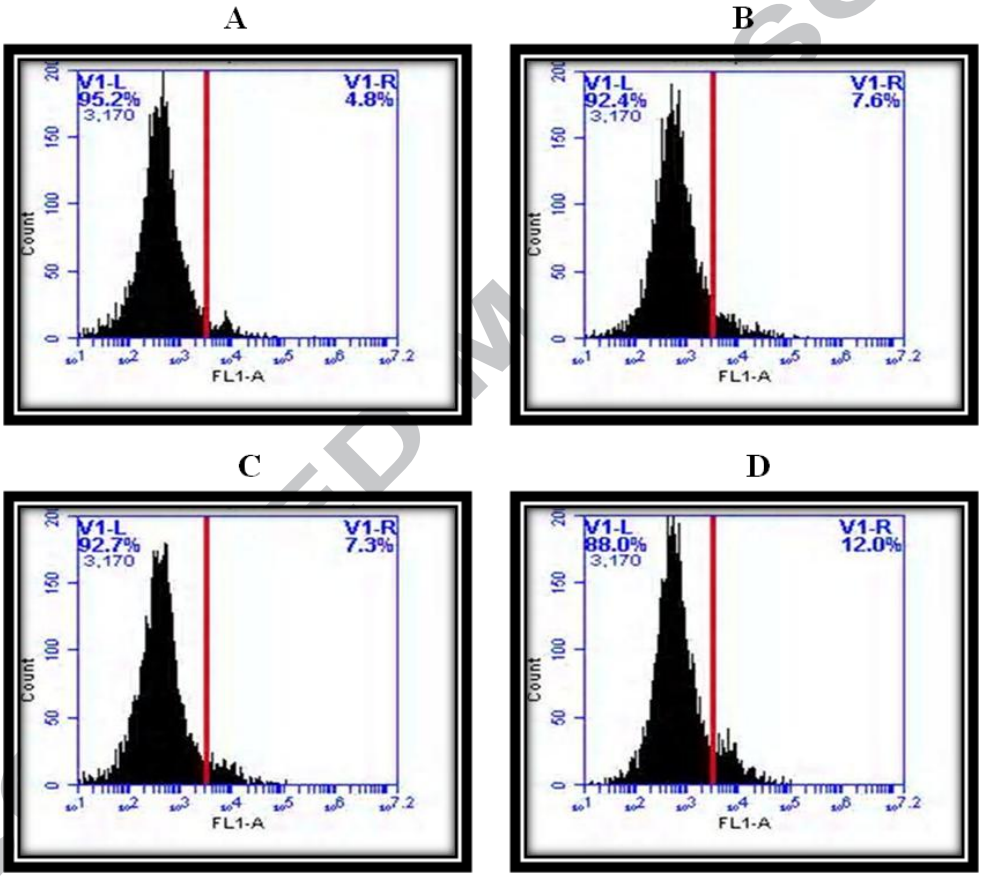


Figure 3: The effect of **6** and **7** on the ROS production in human breast cancer cells (MCF-7); A:Untreated control cells (MCF-7), B: **6** (5 µM), C: **7** (2.5 µM) and D: **7** (5 µM).

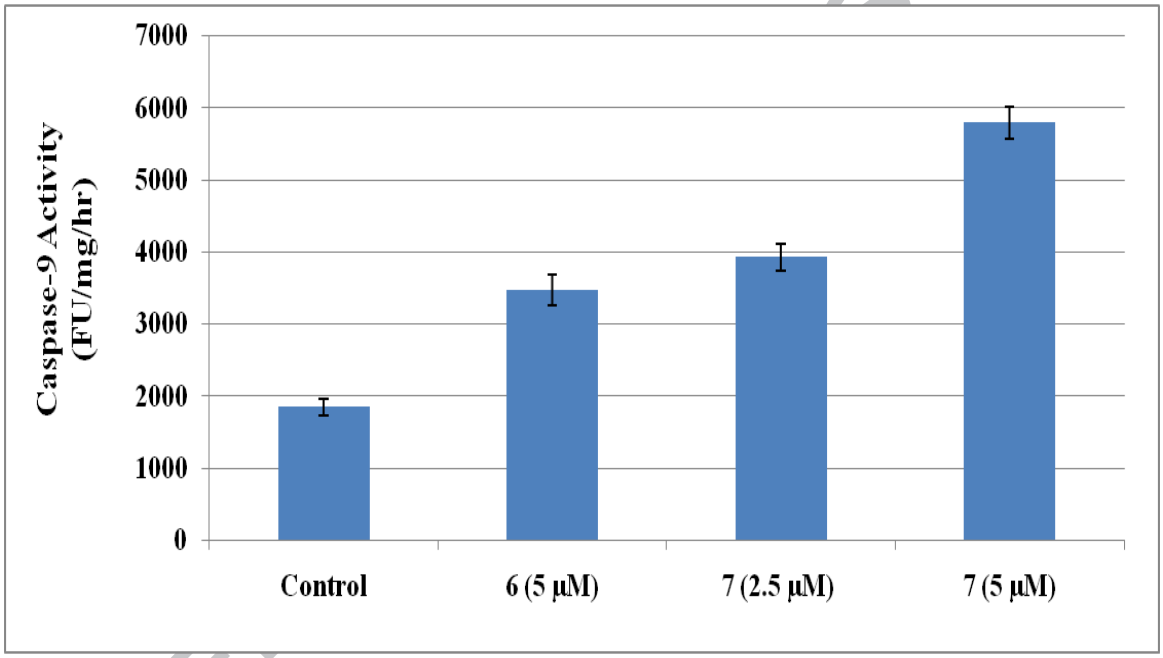


Figure 4. Effect of compounds **6** and **7** on caspase-9 activity.

Graphical Abstract

GRAPHICAL ABSTRACT

Synthesis of ring-C modified oleanolic acid derivatives and their cytotoxic evaluation

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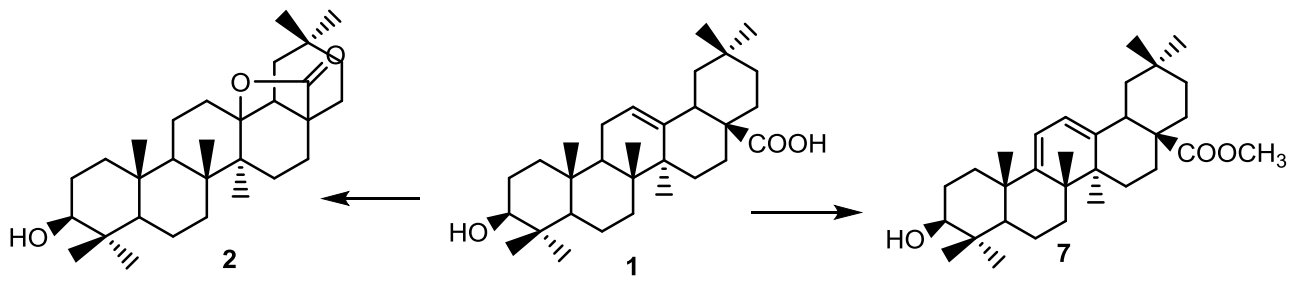

IC_{50} : 2.10±0.65 (A-549); 4.6±0.20 (MCF-7)

IC_{50} : >100 (A-549; MCF-7; ACHN ; Hela)

IC_{50} : 2.96±0.15 (MCF-7); 5.85±0.32 (ACHN); 6.30±0.13 (Hela)

Synthesis of ring-C modified oleanolic acid derivatives and their cytotoxic evaluation

Highlights

- Oleanolic acid ring-C was chemically modified.
- Dienes, α, β-unsaturated ketone, saturated and unsaturated lactones were synthesized.
- The synthesized compounds showed significant anti-cancer activity.
- Compounds **2** and**7** found to active in A-549 and MCF-7cells respectively.
- Compound **7** arrested the cell cycles at G0/G1 phase by inducing apoptosis.