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(22 β ,25R)- 3 β -Hydroxy-spirost-5-en-7-iminoxy-heptanoic acid exhibits anti-prostate cancer activity through caspase pathway

A. A. Hamid^{a,c}, Tanu Kaushal^a, Raghieb Ashraf^b, Arjun Singh^a, Amit Chand Gupta^a, Om Prakash^a, Jayanta Sarkar^b, Debabrata Chanda^a, D.U. Bawankule^a, Feroz Khan^a, Karuna Shanker^a, O.O. Aiyelaagbe^d, Arvind S. Negi^{a*}

^a CSIR-Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Kukrail Picnic Spot Road, P.O. CIMAP, Lucknow-226015, India.

^b CSIR-Central Drug Research Institute (CSIR-CDRI), B.S. 10/1, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow-226031, India.

^c Department of Chemistry, University of Ilorin, Ilorin, Nigeria

^d Organic Chemistry Unit, Department of Chemistry, University of Ibadan, Ibadan, Nigeria.

Abstract

Prostate cancer is one of the most common cancers in men. Diosgenin and related compounds are potential cytotoxic agents. Twelve diverse analogues of long chain fatty acid/ester of diosgenin-7-ketoxime have been prepared. Six of the analogues exhibited significant anticancer activity against a panel of human cancer cell lines with IC₅₀ ranging from 12-35 μ M. Compound **16**, the best representative of the series exerted S phase arrest in DU145 prostate cancer cells and induced apoptosis through caspase pathway. Additionally, these analogues inhibited lipopolysaccharide induced pro-inflammatory cytokines (TNF- α and IL-6) up to 47.7% and 23.3% respectively. Compound **16** was found to be safe in acute oral toxicity in Swiss albino mice up to 300mg/kg dose. The anticancer and antiinflammatory properties of compound **16** are important and can further be optimized for a better anti-prostate cancer candidate.

Keywords: Diosgenin, oxime derivatives, anticancer, caspase pathway, antiinflammatory, acute oral toxicity.

1. Introduction

Steroids are pharmacologically important class of compounds which are biosynthesized through mevalonate pathway in the plants. Due to their crucial role in human physiology and interesting biochemistry, they have attained importance among the researchers [1]. Steroids have an excellent ability to pass through cell membranes and bind to the nuclear and membrane receptors. A small modification in steroid moiety elicits an extensive biological response. Due to their fascinating structural framework and wide range of pharmacological response, steroids have tempted medicinal chemists and biochemists to explore suitable modifications to induce various pharmacological activities. Steroids have been strategically modified as antihormones like antiestrogens, antiprogestins, anti-androgens etc. [2]. Their role as antiinflammatory agents is well established. Steroids have also been modified as anticancer agents where their role as antiproliferative (cytostatic) and cytotoxic is well distinguished [2].

* Corresponding author. Tel.: +91 522 2718583; Fax: +91 522 2342666;
E. mail: arvindcimap@rediffmail.com (as negi) ; CIMAP Communication No. CIMAP/PUB/2016/45

Diosgenin (**1**, Fig. 1) is a steroidal sapogenin present mainly in *Dioscorea* spp. It also occurs in *Costus speciosus*, *Anemarrhena asphodeloides* and in several species of *Smilax*. Owing to its high occurrence (~3-5%) in some plants, it is used as a main precursor for the synthesis of sex hormones, corticosteroids and other steroid products [**3a,b**]. Diosgenin has also been modified to various pharmacologically active analogues. Diosgenin exhibits anticancer [**4a,b**], antiinflammatory [**5**] anti-thrombosis [**6**], enhancing regulatory T-cell immunity [**7**], antiviral [**8**], antidiabetic [**9**], and antimicrobial activities [**10**] etc. In the recent past, diosgenin analogues have attained a special attention for inducing apoptosis in several human carcinomas. The antiproliferative and apoptotic properties of diosgenin are due to its ability to arrest the cell cycle, activate p53, release apoptosis-inducing factor and modulate caspase-3 activity [**11**]. Diosgenin, its saponins and other analogues induce apoptosis mainly prostate cancer cells and colon carcinoma cells [**12a-e**]. Some of the notable anticancer derivatives of diosgenin are Diocin, Timosaponin A-III [**13**], 17-deoxy-26-hydroxy-22-oxo derivative, and several others [**14**].

The present study describes synthesis of some novel analogues of diosgenin at C7-position. Two of the analogues exhibited potential anticancer activity against DU145 prostate cancer cell line. The effect of both the analogues was also checked on production of LPS induced proinflammatory cytokines (TNF- α and IL-6). Compound **16**, the best analogue of the series was further evaluated for cell cycle analysis and mechanistic studies. Finally, *in-vivo* acute oral toxicity of compound **16** was evaluated in Swiss-albino mice.

[Figure 1]

2. Experimental

2.1 General

The starting substrate diosgenin was procured from Sigma-Aldrich USA. All the reagents were analytical grade and were used without any purification. Solvents were dried as per standard procedures and stored under 4Å molecular sieves. Reactions were monitored on Merck precoated silica gel aluminium sheets (TLC, UV254) and compounds were detected under UV light (254nm and 365nm), spraying with 2% ceric sulphate-10% sulphuric acid and heated to 100°C. Purification was done over column silica gel (100-200 mesh, Avra Chemicals, India) eluting with organic solvents. Melting points were determined on E-Z Melt MP apparatus in open capillaries (Stanford Research System, USA) and were uncorrected. NMR experiments were performed on Bruker Avance (300MHz/500MHz) spectrometer with TMS as internal standard (δ , 0.00 ppm). Electrospray Ionisation mass spectra were recorded on API3000 LC-MS-MS (Applied Biosystem, USA) after dissolving the compounds in methanol or acetonitrile. High Resolution Mass (HRMS) were recorded on Agilent 6520Q-TOF after dissolving the compounds in methanol. FT-IR spectra were recorded on Perkin-Elmer SpectrumBX. Purity of compounds was determined on Waters ACQUITY UPLC BEH130Å system. Nomenclature of steroid derivatives has been given as per the recommendations by the Joint Commission on the Biochemical Nomenclature (JCBN) of IUPAC [**15**].

2.2. Chemical synthesis

2.2.1. Synthesis of (22 β ,25R)-spirost-5-en-3 β -yl-3-acetate (**2**)

Diosgenin (1g, 2.4mmol) was taken in dry chloroform (10mL). To this stirred solution, DMAP (50mg) and acetic anhydride (1mL, 10.5mmol) were added and further stirred for 2h at RT. The reaction mixture was diluted with water and extracted with chloroform, washed with dil. HCl (5%, 10mL) and washed with water. Organic layer was dried over anhydrous sodium sulphate and evaporated to dryness. Residue was recrystallised with chloroform-hexane (1:3) to get compound **2** as white amorphous solid.

2: Yield=93%, mp=193-95°C [195°C, **16**]; ^1H NMR (CDCl_3), δ 0.77 (s, 3H, 18- CH_3), 0.96 (d, 3H, 27- CH_3 , $J=6.9\text{Hz}$), 1.02 (s, 3H, 19- CH_3), 1.11-2.31 (m, 25H, rest of the 1x CH_3 , 8x CH_2 and 6xCH of steroidal ring), 2.01 (s, 3H, CH_3COO , Acetate), 2.24-2.31 (bd, 2H, 7- CH_2), 3.38 (m, 2H, 26- CH_2), 4.37 (bs, 1H, 3-CH), 4.42 (bd, 1H, 16-CH), 5.36 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.89 (C21), 16.64 (C18), 19.69 (C19), 21.20 (C11), 21.74 (acetate CH_3), 28.12 (C24), 29.19 (C2), 30.66 (C25), 31.78 (C23), 31.80 (C8), 32.21 (C7), 32.41 (C15), 37.10 (C10), 37.34 (C1), 38.47 (C12), 40.10 (C4), 40.63 (C13), 42.00 (C20), 42.68, 50.35 (C9), 56.82 (C14), 62.52 (C17), 67.19 (C26), 74.26 (C3), 81.16 (C16), 109.60 (C22), 122.72 (C6), 140.05 (C5), 170.82 (acetate ester). ESI Mass (MeOH) for $\text{C}_{29}\text{H}_{44}\text{O}_4$: 457.3 $[\text{M}+\text{H}]^+$, 479.3 $[\text{M}+\text{Na}]^+$, 495.4 $[\text{M}+\text{K}]^+$. IR (KBr, cm^{-1}): 2907, 1724, 1451, 1231.

2.2.2. Synthesis of (22 β ,25R)-spirost-5-en-7-oxo-3 β -yl-3-acetate (**3**)

A mixture of dry methylene chloride (25mL) and dry pyridine (10mL) was stirred with cooling (5-10°C). To this crushed chromium trioxide (1g) was added and further stirred for 10 min. To this reaction mixture diosgenin 3-acetate (**2**, 500mg, 1.09mmol) was added as a solution in methylene chloride (3-5mL) drop-wise. It was stirred for 30min in ice-bath and then at room temperature for 4 hours. On completion methanol (2mL) was added to decompose the excess of chromic acid. Solvent was evaporated and water was added to it. The reaction mixture was extracted with ethyl acetate, organic layer was washed with water, dries over anhydrous sodium sulphate and evaporated. The crude mass was purified through silica gel column eluting with hexane-ethyl acetate to get the desired 7-ketone derivative (**3**) as creamish white solid at 8% ethyl acetate-hexane.

3: Yield= 351g (68%), mp=179-182°C; ^1H NMR (CDCl_3), δ 0.78 (s, 3H, 18- CH_3), 0.97 (d, 3H, 27- CH_3 , $J=6.6\text{Hz}$), 1.14 (s, 3H, 19- CH_3), 1.16-2.84 (m, 23H, rest of the 1x CH_3 , 7x CH_2 and 6xCH of steroidal ring), 2.04 (s, 3H, CH_3COO , 3-acetate), 3.39 (m, 2H, 26- CH_2), 4.46 (bs, 1H, 3-CH), 4.71 (bs, 1H, 16-CH), 5.71 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 15.02 (C21), 16.79 (C18), 17.50 (C27), 17.64 (C19), 21.29 (C11), 21.59 (acetate CH_3), 27.72 (C24), 29.20 (C2), 30.70 (C23), 31.84 (C23), 34.09 (C15), 36.39 (C1), 38.18 (C12), 38.84 (C10), 39.05 (C4), 41.36 (C13), 41.98 (C20), 45.29 (C8), 49.87 (C14), 50.07 (C9), 61.57 (C17), 67.17 (C26), 72.53 (C3), 81.13 (C16), 109.55 (C22), 126.87 (C6), 164.43 (C5), 170.61 (acetate ester), 201.65 (C7). ESI Mass (MeOH) for $\text{C}_{29}\text{H}_{42}\text{O}_5$: 471.4 $[\text{M}+\text{H}]^+$, 493.4 $[\text{M}+\text{Na}]^+$, 509.3 $[\text{M}+\text{K}]^+$. IR (KBr, cm^{-1}): 2907, 1724, 1451, 1231.

2.2.3. Synthesis of (22 β ,25R)-7-(hydroxyimino)-spirost-5-en-3 β -yl-3-acetate (**4**)

Diosgenin 7-ketone (**3**, 500mg, 1.06mmol) was taken in dry pyridine (6mL). To this hydroxylamine hydrochloride (150mg, 2.16mmol) was added and refluxed for 2h. On completion, water was added to it, acidified with HCl (5%, 10mL), extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulphate. The organic layer was dried *in vacuo* to get oxime **4** as oil.

4: Yield=494mg (96%), oil; ^1H NMR (CDCl_3), δ 0.77 (s, 3H, 18- CH_3), 0.97 (d, 3H, 27- CH_3 , $J=6.6\text{Hz}$), 1.11 (s, 3H, 19- CH_3), 1.20-2.41 (m, 21H, rest of the 1 $\times\text{CH}_3$, 7 $\times\text{CH}_2$ and 6 $\times\text{CH}$ of steroidal ring), 2.01 (s, 3H, CH_3COO , 3-acetate), 2.41-2.43 (m, 3H), 2.82 (bs, 1H), 3.44 (m, 2H, 26- CH_2), 4.56 -4.68 (m, 2H, 3-CH & 16-CH), 6.57 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.97 (C21), 17.08 (C18), 17.46 (C27), 18.28 (C19), 20.88 (C11), 21.67 (C3-acetate CH_3), 27.85 (C24), 29.61 (C2), 30.64 (C25), 31.71 (C23), 34.88 (C15), 35.16 (C1), 38.43 (C12), 38.85 (C10), 39.00 (C4), 36.66 (C8), 41.01 (C13), 41.96 (C20), 49.80 (C14), 50.26 (C9), 60.98 (C17), 67.11 (C26), 73.38 (C3), 81.28 (C16), 114.21 (C6), 109.96 (C22), 151.60 (C5), 156.74 (C7), 170.73 (C3, acetate ester). ESI Mass (MeOH) for $\text{C}_{29}\text{H}_{43}\text{NO}_5$: 486 $[\text{M}+\text{H}]^+$, 508 $[\text{M}+\text{Na}]^+$; Negative mode: 484 $[\text{M}-\text{H}]^-$. ESI-HRMS for $\text{C}_{29}\text{H}_{44}\text{NO}_5$ $[\text{M}+\text{H}]^+$, calc. 486.3219, found 486.3197.

2.2.4. General synthesis of fatty acid esters (**5-10**)

Synthesis of (22 β ,25R)- 3 β -acetoxy-spirost-5-en-7-iminoxy-ethylacetate (**5**)

Diosgenin 7-keto oxime (**4**, 200mg, 0.41mmol) was taken in dry acetone (15mL) and anhydrous potassium carbonate (1g). To this ethyl iodoacetate (0.1mL, 181mg, 0.84mmol) was added and the reaction mixture was refluxed for 4h. On completion, potassium carbonate was filtered out and filtrate was evaporated. The residue was dissolved in ethyl acetate and washed with water. The organic layer was dried over anhydrous sodium sulphate and evaporated. The residue thus obtained was purified through filter column of silica gel eluting with ethyl acetate-hexane. The desired ester **4** was obtained at 6% ethyl acetate-hexane as white amorphous solid.

5: Yield= 210mg (89%), mp=146-148 $^\circ\text{C}$; ^1H NMR (CDCl_3), δ 0.77 (s, 3H, 18- CH_3), 0.98 (d, 3H, 27- CH_3 , $J=6.9\text{Hz}$), 1.12 (s, 3H, 19- CH_3), 1.33 (t, 3H, CH_3 , ethyl, $J=8.0\text{Hz}$), 1.20-2.51 (m, 24H, rest of the 1 $\times\text{CH}_3$, 8 $\times\text{CH}_2$ and 5 $\times\text{CH}$ of steroidal ring), 2.02 (s, 3H, CH_3COO , 3-acetate), 3.42 (m, 2H, 26- CH_2), 4.20 (q, 2H, OCH_2 , ethyl, $J=7.1\text{Hz}$), 4.54 (s, 2H, OCH_2), 4.56 (m, 2H, 3-CH & 16-CH), 6.54 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.65 (C21), 15.05 (CH_3 , ethyl), 16.99 (C18), 17.51 (C27), 18.28 (C19), 20.94 (C11), 21.68 (C3-acetate CH_3), 27.82 (C24), 29.21 (C2), 30.70 (C25), 31.82 (C23), 34.92 (C15), 36.61 (C1), 38.42 (C12), 38.81 (C10), 39.11 (C4), 36.66 (C8), 41.11 (C13), 41.96 (C20), 49.85 (C14), 50.50 (C9), 61.51 (C17), 61.13 (CH_2 , ethyl), (C26), 71.01 (2'- CH_2), 73.20 (C3), 81.08 (C16), 114.48 (C6), 109.58 (C22), 153.33 (C5), 157.70 (C7), 170.66 (C3, acetate ester), 170.66 (ester). ESI Mass (MeOH) for $\text{C}_{33}\text{H}_{49}\text{NO}_7$: 572.8 $[\text{M}+\text{H}]^+$, 595.7 $[\text{M}+\text{Na}]^+$, 611.6 $[\text{M}+\text{K}]^+$; IR (KBr, cm^{-1}): 2947, 1737, 1638, 1451, 1241, .

2.2.5. (22 β ,25R)- 3 β -acetoxy-spirost-5-en-7-iminoxy-ethylpropionate (**6**)

There were some isomeric CH peaks in ^{13}C indicating isomeric mixture.

6: Yield=198mg (82%), mp=141-143°C; ^1H NMR (CDCl_3), δ 0.75 (s, 3H, 18- CH_3), 0.95 (d, 3H, 27- CH_3 , $J=6.9\text{Hz}$), 1.13 (s, 3H, 19- CH_3), 1.30 (t, 3H, CH_3 , ethyl ester, $J=7.8\text{Hz}$), 1.22-2.82 (m, 27H, rest of the 1 $\times\text{CH}_3$, 9 $\times\text{CH}_2$ and 6 $\times\text{CH}$ of steroidal ring), 2.00 (s, 3H, CH_3COO , 3-acetate), 3.37-3.42 (m, 2H, 26- CH_2), 4.15 (q, 2H, OCH_2 , ethyl ester, $J=4.7\text{Hz}$), 4.19 (m, 2H, -N- OCH_2), 4.64 (m, 2H, 3-CH & 16-CH), 6.52 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.69 (C21), 15.04 (CH_3 , ethyl), 16.95 (C18), 17.50 (C27), 18.23 (C19), 20.91 (C11), 21.64 (C3-acetate CH_3), 27.81 (C24), 29.21 (C2), 30.67 (C25), 31.80 (C23), 34.98 (C15), 36.55 (C2'), 36.66 (C1), 38.40 (C12), 38.83 (C10), 39.09 (C4), 36.66 (C8), 41.14 (C13), 41.92 (C20), 49.85 (C14), 50.42 (C9), 61.57 (C17), 61.03 (CH_2 , ethyl), 67.13 (C26), 73.14 and 73.24 (C3), 81.05 and 81.09 (C16), 114.46 and 114.74 (C6), 109.48 (C22), 153.08 (C5), 156.90 (C7), 173.31 (C3, acetate ester), 170.66 (ester). ESI Mass (MeOH) for $\text{C}_{34}\text{H}_{51}\text{NO}_7$: 586 $[\text{M}+\text{H}]^+$, 608 $[\text{M}+\text{Na}]^+$, 644 $[\text{M}+\text{K}]^+$; IR (KBr, cm^{-1}): 2948, 1738, 1638, 1458, 1241.

2.2.6. (22 β ,25R)- 3 β -acetoxyspirost-5-en-7-iminoxy-ethylbutyrate (**7**)

7: Yield=205mg (83%), mp=145-148°C; ^1H NMR (CDCl_3), δ 0.77 (s, 3H, 18- CH_3), 0.97 (d, 3H, 27- CH_3 , $J=6.9\text{Hz}$), 1.12 (s, 3H, 19- CH_3), 1.32 (bs, 3H, CH_3 , ethyl ester), 1.21-2.90 (m, 29H, rest of the 1 $\times\text{CH}_3$, 10 $\times\text{CH}_2$ and 6 $\times\text{CH}$ of steroidal ring), 2.03 (s, 3H, CH_3COO , Acetate), 3.46 (m, 2H, 26- CH_2), 4.12 (q, 2H, OCH_2 , ethyl, $J=7.1\text{Hz}$), 4.05 (t, 2H, -N- OCH_2 , $J=7.2\text{Hz}$), 4.42 (m, 1H, 3-CH), 4.60 (m, 1H, 16-CH), 6.45 (s, 1H, 6-CH). ESI Mass (MeOH) $\text{C}_{35}\text{H}_{53}\text{NO}_7$: 600.9 $[\text{M}+\text{H}]^+$, 622.8 $[\text{M}+\text{Na}]^+$, 638.7 $[\text{M}+\text{K}]^+$; IR (KBr, cm^{-1}): 2928, 1737, 1637, 1459, 1241.

2.2.7. (22 β ,25R)- 3 β -acetoxyspirost-5-en-7-iminoxy-ethylpentanoate (**8**)

8: Yield= 176mg (70%), mp=199-202°C; ^1H NMR (CDCl_3), δ 0.76 (s, 3H, 18- CH_3), 0.97 (d, 3H, 27- CH_3 , $J=6.9\text{Hz}$), 1.19 (s, 3H, 19- CH_3), 1.23 (t, 3H, CH_3 , ethyl ester, $J=7.1\text{Hz}$), 1.21-2.84 (m, 31H, rest of the 1 $\times\text{CH}_3$, 8 $\times\text{CH}_2$ and 6 $\times\text{CH}$), 2.02 (s, 3H, CH_3COO , 3-acetate), 3.44 (m, 2H, 26- CH_2), 4.06-4.13 (m, 4H, OCH_2 of ethyl and N- OCH_2), 4.54 (m, 1H, 3-CH), 4.66 (m, 1H, 16-CH), 6.56 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.55 (C21), 14.98 (CH_3 , ethyl), 17.04 (C18), 17.45 (C27), 18.28 (C19), 20.88 (C11), 21.65 (C3-acetate CH_3), 27.85 (C24), 29.12 (C2), 30.66 (C25), 31.75 (C23), 34.88 (C15), 36.67 (C2'), 37.57 (C1), 38.45 (C12), 38.87 (C10), 39.02 (C4), 36.66 (C8), 30.66 (C25), 41.04 (C13), 41.96 (C20), 49.84 (C14), 50.30 (C9), 61.15 (C17), 60.76 (CH_2 , ethyl), 60.76 (5' CH_2), 67.13 (C26), 73.34 (C3), 81.22 (C16), 114.08 (C6), 109.84 (C22), 151.85 (C5), 156.99 (C7), 171.54 (C3, acetate ester), 170.67 (ester). ESI Mass (MeOH) for $\text{C}_{36}\text{H}_{55}\text{NO}_7$: 636.7 $[\text{M}+\text{Na}]^+$; 652.9 $[\text{M}+\text{K}]^+$.

2.2.8. (22 β ,25R)- 3 β -acetoxyspirost-5-en-7-iminoxy-ethylhexanoate (**9**)

9: Yield=217mg (84%), mp=89-91°C; ^1H NMR (CDCl_3), δ 0.72 (s, 3H, 18- CH_3), 0.92 (d, 3H, 27- CH_3 , $J=6.9\text{Hz}$), 1.11 (s, 3H, 19- CH_3), 1.19 (t, 3H, CH_3 , ethyl ester, $J=7.1\text{Hz}$), 1.21-2.93 (m, 33H, rest of the 1 $\times\text{CH}_3$, 12 $\times\text{CH}_2$ and 6 $\times\text{CH}$), 2.01 (s, 3H, CH_3COO , 3-acetate), 3.34 (m, 2H, 26- CH_2), 4.04-4.09 (q, 4H, OCH_2 of ethyl and N- OCH_2 , $J=7.1\text{Hz}$), 4.46 (m, 1H, 3-CH), 4.60 (m, 1H, 16-CH), 6.52 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.47, 14.97, 16.95, 17.41, 18.19, 20.80, 21.32, 21.59, 27.76, 28.82, 31.67, 32.10, 34.63, 36.61, 37.00, 37.20, 37.62, 38.40, 38.88, 38.95, 40.96, 41.89, 49.80, 49.88,

54.12, 60.84, 61.15, 67.11, 70.36, 73.40, 81.08, 109.89, 114.33, 152.32, 157.16, 170.91, 171.80. ESI Mass (MeOH) for $C_{37}H_{57}NO_7$: 628.3 $[M+H]^+$;

2.2.9. (22 β ,25R)- 3 β -acetoxy-spirost-5-en-7-iminoxy-ethylheptanoate (**10**)

10: Yield=243mg (92%), oil; 1H NMR ($CDCl_3$), δ 0.78 (s, 3H, 18- CH_3), 0.96 (d, 3H, 27- CH_3 , $J=7.0$ Hz), 1.11 (s, 3H, 19- CH_3), 1.23 (t, 3H, CH_3 , ethyl, $J=7.5$ Hz), 1.23-2.39 (m, 35H, rest of the 1 \times CH_3 , 13 \times CH_2 and 6 \times CH), 2.02 (s, 3H, CH_3COO , 3-acetate), 3.39 (m, 2H, 26- CH_2), 4.08-4.13 (m, 4H, OCH_2 of ethyl and N-O- CH_2 , $J=8.0$ Hz, 5.3Hz), 4.46 (m, 1H, 3- CH), 4.60 (m, 1H, 16- CH), 6.45 (s, 1H, 6- CH); ^{13}C NMR ($CDCl_3$, 75 MHz); δ 14.26, 14.68, 16.63, 17.12, 17.95, 20.57, ESI Mass (MeOH) for $C_{38}H_{59}NO_7$: 664.7 $[M+Na]^+$; 681.3 $[M+K]^+$; ESI-HRMS for $C_{38}H_{60}NO_7$ calc:642.4370, observed:642.4360; IR (KBr, cm^{-1}): 2929, 1735, 1460, 1176.

2.2.10. Saponification of esters (**5-10**) to get acid derivatives (**11-16**)

Synthesis of (22 β ,25R)- 3 β -hydroxy-spirost-5-en-7-iminoxy-ethanoic acid (**11**)

Ester **6** (100mg, 0.17mmol) was taken in 1.2% aqueous methanolic alkali (water:MeOH=1:9). The reaction mixture was stirred at room temperature for 2h. Solvent was removed *in vacuo* and water was added, acidified with dil. HCl (5%, 10mL), extracted with ethyl acetate. Organic layer was washed with water, dried over sodium sulphate and evaporated. The residue thus obtained was recrystallised with chloroform:hexane (1:2) to get **11** as solid.

11: Yield=87mg (91%), mp=145-146°C; 1H NMR ($CDCl_3$), δ 0.78 (s, 3H, 18- CH_3), 0.95 (d, 3H, 27- CH_3 , $J=6.9$ Hz), 1.14 (s, 3H, 19- CH_3), 1.19-2.62 (m, 25H, rest of the 1 \times CH_3 , 8 \times CH_2 and 6 \times CH of steroidal ring), 3.42 (m, 2H, 26- CH_2), 4.54 (s, 2H, N- OCH_2), 4.56 (m, 2H, 3- CH & 16- CH), 6.54 (s, 1H, 6- CH). ESI Mass (MeOH) for $C_{29}H_{43}NO_6$: 502.4 $[M+H]^+$, 524.3 $[M+Na]^+$, 540.4 $[M+K]^+$; IR (KBr, cm^{-1}): 2947, 1638, 1451, 1241, .

2.2.11. (22 β ,25R)- 3 β -acetoxy-spirost-5-en-7-iminoxy-propanoic acid (**12**)

12: Yield=82 (86%), mp=114-116°C; 1H NMR ($CDCl_3$), δ 0.72 (s, 3H, 18- CH_3), 0.92 (d, 3H, 27- CH_3 , $J=6.6$ Hz), 1.22 (s, 3H, 19- CH_3), 1.27-2.66 (m, 27H, rest of the 1 \times CH_3 , 9 \times CH_2 and 6 \times CH of steroidal ring), 2.11 (s, 3H, CH_3COO , Acetate), 3.38 (m, 2H, 26- CH_2), 3.79 (bs, 2H, N-O- CH_2), 4.13 (m, 1H, 3- CH), 4.32 (m, 1H, 16- CH), 6.46 (s, 1H, 6- CH). ^{13}C NMR ($CDCl_3$, 75 MHz); δ 14.63 (C21), 16.91 (C18), 17.46 (C27), 18.28 (C19), 20.92 (C11), 21.96 (C3-acetate CH_3), 29.15 (C24), 29.58 (C2), 30.62 (2'- CH_2), 30.62 (C25), 31.50 (C8), 31.75 (C23), 32.06 (C15), 37.59 (C1), 38.78 (C12), 40.98 (C4), 41.10 (C13), 41.89 (C20), 49.99 (C9), 54.24 (C14), 61.05 (3'- CH_2), 62.31 (C17), 67.09 (C26), (2'- CH_2), 71.12 (C3), 81.05 (C16), 113.54 (C6), 109.49 (C22), 156.9 (C5), 160 (C7), 171 (C3, acetate ester), 173.51 (COOH); ESI Mass (MeOH) for $C_{32}H_{47}NO_7$: 580.2 $[M-Na]^+$.

2.2.12. (22 β ,25R)- 3 β -hydroxy-spirost-5-en-7-iminoxy-butanoic acid (**13**)

13: Yield=73 (83%), mp=147-149°C; ^1H NMR (CDCl_3), δ 0.68 (s, 3H, 18- CH_3), 0.90 (d, 3H, 27- CH_3 , $J=6.9\text{Hz}$), 1.11 (s, 3H, 19- CH_3), 1.15-2.57 (m, 29H, rest of the 1 $\times\text{CH}_3$, 10 $\times\text{CH}_2$ and 6 $\times\text{CH}$ of steroidal ring), 3.42 (t, 2H, 26- CH_2 , $J=6.0\text{Hz}$), 4.05 (t, 2H, N- OCH_2 , $J=6.3\text{Hz}$), 4.56 (m, 2H, 3-CH & 16-CH), 6.54 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.65 (C21), 15.05 (CH_3 , ethyl), 16.99 (C18), 17.51 (C27), 18.28 (C19), 20.94 (C11), 27.82 (C24), 29.21 (C2), 30.70 (C25), 31.82 (C23), 34.92 (C15), 36.61 (C1), 38.42 (C12), 38.81 (C10), 39.11 (C4), 36.66 (C8), 41.11 (C13), 41.96 (C20), 49.85 (C14), 50.50 (C9), 61.51 (C17), 61.13 (CH_2 , ethyl), (C26), 71.01 (2'- CH_2), 73.20 (C3), 81.08 (C16), 114.48 (C6), 109.58 (C22), 153.33 (C5), 157.70 (C7), 174.81 (Acetate) 178.47 (COOH). ESI Mass (MeOH) for $\text{C}_{31}\text{H}_{47}\text{NO}_6$: 529.4 [M^+].

2.2.13. (22 β ,25 R)-3 β -hydroxy-spirost-5-en-7-iminoxy-pentanoic acid (**14**)

14: Yield=79mg (89%), mp=159-162°C; ^1H NMR (CDCl_3), δ 0.68 (s, 3H, 18- CH_3), 0.90 (d, 3H, 27- CH_3 , $J=5.4\text{Hz}$), 1.11 (s, 3H, 19- CH_3), 1.15-2.57 (m, 31H, rest of the 1 $\times\text{CH}_3$, 11 $\times\text{CH}_2$ and 6 $\times\text{CH}$ of steroidal ring), 3.42 (t, 2H, 26- CH_2 , $J=6.3\text{Hz}$), 4.54 (s, 2H, N- OCH_2), 4.56 (m, 2H, 3-CH & 16-CH), 6.54 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 14.65 (C21), 15.05 (CH_3 , ethyl), 16.99 (C18), 17.51 (C27), 18.28 (C19), 20.94 (C11), 27.82 (C24), 29.21 (C2), 30.70 (C25), 31.82 (C23), 34.92 (C15), 36.61 (C1), 38.42 (C12), 38.81 (C10), 39.11 (C4), 36.66 (C8), 41.11 (C13), 41.96 (C20), 49.85 (C14), 50.50 (C9), 61.51 (C17), 61.13 (CH_2 , ethyl), (C26), 71.01 (2'- CH_2), 73.20 (C3), 81.08 (C16), 114.48 (C6), 109.58 (C22), 153.33 (C5), 157.70 (C7), 178.47 (COOH). ESI Mass (MeOH) for $\text{C}_{34}\text{H}_{51}\text{NO}_7$: 544.3 [$\text{M}+\text{H}$] $^+$; IR (KBr, cm^{-1}): 2947, 1737, 1638, 1451, 1241, .

2.2.14. (22 β ,25 R)-3 β -hydroxy-spirost-5-en-7-iminoxy-hexanoic acid (**15**)

15: Yield=87mg (91%), mp=155-157°C; ^1H NMR (CDCl_3), δ 0.72 (s, 3H, 18- CH_3), 0.93 (d, 3H, 27- CH_3 , $J=6.6\text{Hz}$), 1.13 (s, 3H, 19- CH_3), 1.18-2.78 (m, 24H, rest of the 1 $\times\text{CH}_3$, 8 $\times\text{CH}_2$ and 6 $\times\text{CH}$ of steroidal ring), 3.42 (m, 2H, 26- CH_2), 3.44 (t, 2H, N- OCH_2 , $J=6.3\text{Hz}$), 4.33 (m, 2H, 3-CH & 16-CH), 6.41 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 75 MHz); δ 13.72, 15.53, 16.16, 16.97, 19.39, 23.64, 24.33, 28.46, 29.13, 30.16, 30.30, 31.27, 32.86, 33.49, 35.70, 36.12, 37.33, 37.62, 37.99, 41.25, 48.72, 48.99, 50.27, 60.21, 60.46, 65.52, 69.20, 79.55, 107.93, 111.81, 151.61, 155.01, 172.84; ESI Mass (MeOH) for $\text{C}_{33}\text{H}_{51}\text{NO}_6$: 558.3 [$\text{M}+\text{H}$] $^+$.

2.2.15. (22 β ,25 R)-3 β -hydroxy-spirost-5-en-7-iminoxy-heptanoic acid (**16**)

16: Yield=80mg (84%), mp=237-239°C; ^1H NMR (CDCl_3), δ 0.77 (s, 3H, 18- CH_3), 0.97 (d, 3H, 27- CH_3 , $J=7.0\text{Hz}$), 1.14 (s, 3H, 19- CH_3), 1.24-2.45 (m, 35H, rest of the 1 $\times\text{CH}_3$, 13 $\times\text{CH}_2$ and 6 $\times\text{CH}$ of steroidal ring), 3.46 (bs, 2H, 26- CH_2), 4.02 (t, 2H, N- OCH_2 , $J=6.5\text{Hz}$), 4.46 (m, 2H, 3-CH & 16-CH), 6.43 (s, 1H, 6-CH). ^{13}C NMR (CDCl_3 , 100MHz); δ 14.67, 16.64, 18.06, 20.65, 22.69, 24.77, 25.62, 28.81, 28.88, 29.15, 29.36, 30.30, 31.22, 34.03, 34.60, 36.59, 37.22, 38.34, 38.87, 40.73, 41.60, 42.14, 49.73, 50.31, 61.09, 66.84, 71.04, 73.52, 80.84, 109.35, 113.43, 153.06, 155.71, 178.77. ESI Mass (MeOH) for $\text{C}_{34}\text{H}_{53}\text{NO}_6$: 594.7 [$\text{M}+\text{Na}$] $^+$, 610.5 [$\text{M}+\text{K}$] $^+$; Negative mode: 570.9 [$\text{M}-\text{H}$] $^-$. ESI-HRMS calc for $\text{C}_{34}\text{H}_{54}\text{NO}_6$: 572.3914, found, 572.3912; IR (KBr, cm^{-1}): 2947, 1737, 1638, 1451, 1241,

2.2.16. (22 β ,25R)-7-Oxo-spirosta-3,5-diene (**17**)

Benzyltriphenylphosphonium bromide (Wittig salt, 1g, 2.1mmol) was stirred in dry toluene (15mL). To this sodium hydride (100mg, 4mmol) was added and further stirred for 10 min. 7-Keto derivative **3** (250mg, 0.53mmol) was added to it and reaction mixture was refluxed for 2h. On completion water (20mL) was added to it and reaction mixture was extracted with ethyl acetate, organic layer was washed with water, dries over anhydrous sodium sulphate and evaporated. The residue thus obtained was recrystallised with chloroform-hexane (1:4) mixture to get an exclusive product (**17**) was obtained as creamish white solid. Reaction was done with several other wittig salts of benzyl bromides (4-nitro; 3,4,5-trimethoxy; 3,4-methylenedioxy) yielding always the same product **17**.

17: Yield=78mg (89%), mp=182-185°C; ¹H NMR (CDCl₃), δ 0.76 (s, 3H, 18-CH₃), 0.95 (d, 3H, 27-CH₃, J=6.9Hz), 1.14 (s, 3H, 19-CH₃), 1.19-2.29 (m, 19H, rest of the 1xCH₃, 5xCH₂ and 6xCH of steroidal ring), 2.52 (t, 1H, 2-CH₂, J=6.3Hz), 2.87 (t, 1H, 2-CH₂), 3.38 (m, 2H, 26-CH₂), 4.41 (q, 1H, 16-CH, J=4.6Hz), 5.58 (s, 1H, 6-CH), 6.14 (m, 2H, 3-CH and 4-CH). ¹³C NMR (CDCl₃, 75 MHz); δ 15.02, 16.86, 16.97, 17.50, 21.31, 23.76, 29.19, 30.69, 31.83, 33.20, 34.27, 36.80, 39.31, 41.79, 42.01, 45.96, 49.94, 50.61, 61.60, 67.15, 81.41, 109.51, 124.32, 128.05, 137.13, 161.59, 202.14 ; ESI Mass (MeOH) for C₂₇H₃₈O₃: 411.2 [M+H]⁺; ESI-HRMS for C₂₇H₃₉O₃ [M+H]⁺, calc. 411.2899, found 411.2872.

2.3. Purity profile of analogues **9**, **10**, **11**, **13**, **14** and **16**

The ACQUITY UPLC BEH130Å (2.1×100mm, 1.7 μ m) C₁₈ column (Waters, USA) thermostated at 25±0.1°C and fitted in ACQUITY UPLC H-Class Bio System was used for purity determination of the compounds prior to *in-vitro* & *in-vivo* assay. UPLC analysis was performed with a two-solvent system using isocratic elution of acetonitrile/methanol and water (*mobile phase-I*: Water: CH₃CN- 70:30; *mobile phase-II*: Water: CH₃OH- 80:20; run time-12min; flow rate- 0.3 ml/min). The injection volume was 2 μ l. The column elution was monitored in the range of 190-400nm using photodiode array detector (A16UPD0038, ACQUITY Waters, Cell volume- 500nl). Purity of the compounds was calculated using UPLC peak area normalization method. UPLC provides improved resolution and higher throughput as compared to traditional reverse phase-HPLC system. In the absence of primary standards, peak area normalization method was adopted to calculate the chromatographic purity of respective compounds [17].

2.4. Pharmacology

2.4.1. Cell Culture

Human cancer cell lines, FaDU (Hypopharyngeal carcinoma), A549 (lung carcinoma), DLD1 (colorectal adenocarcinoma), MCF-7 (Breast adenocarcinoma), DU145 (Prostate carcinoma) and PC3 (Prostate carcinoma) were originally obtained from American type of cell culture collection (ATCC), USA and grown at 37°C in DMEM supplemented with 10% FBS and Ab-Am (antibiotic-antimitotic) solution in a CO₂ incubator (New Brunswick/Eppendorff, Germany) under 5% CO₂ and 95% relative

humidity. Tamoxifen, doxorubicin, and podophyllotoxin were used as standard anticancer drugs (positive control) for cytotoxicity.

2.4.2. Cytotoxicity evaluation by Sulphorhodamine assay

Cytotoxicity of diosgenin derivatives was evaluated by Sulphorhodamine B assay [18]. In brief, in 96-well culture plates, 10^4 cells/well were added and incubated overnight at 37°C in 5% CO₂. Next day (at 80% confluency), serial dilutions of test compound were added to the wells. Untreated cells served as control. After 48h, cells were fixed with ice-cold 50% (w/v) trichloroacetic acid (100µL/well) for 1h at 4°C. Cells were then stained with SRB dye (0.4% w/v in 1% acetic acid, 50µL/well), washed and air-dried. Bound dye was solubilised with 10mM Tris base (150µL/well) and absorbance was read at 540nm on a plate reader (Biotek, USA). The cytotoxic effect was calculated as;

% inhibition in cell growth= $[1 - (\text{Absorbance of treated cells} / \text{Absorbance of untreated cells}) \times 100]$.
Determination of 50% inhibitory concentration (IC₅₀) was based on dose-response curves.

2.4.3. Cell cycle analysis

The effect of most potent compound **16** on cell division cycle was assessed by flow cytometry with PI-stained cellular DNA, as described earlier [19]. Briefly, in 6-well culture plate, 4×10^5 cells per well were seeded and grown overnight at 37°C in 5% CO₂. After treating with compound **16** for different time points, cells were harvested by trypsinization and fixed with ice-cold 70% ethanol for 30min at 4°C. The pellets were washed with PBS and re-suspended in a solution containing propidium iodide (20mg/mL), TritonX100 (0.1%) and RNase (1mg/mL) in PBS. After distribution of cells in different phases of cell cycle, "Cell Quest" software was used for calculations.

2.4.4. Western blot assay

Compound **16** treated cells were lysed (30min, in ice) with cold M-PER (mammalian protein extraction reagent) supplemented with protease inhibitor cocktail. Equal amount of proteins (25µg) extracted from the cells treated at different time intervals were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred electrophoretically onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk powder dissolved in Tris-buffer saline (TBS; 20mM Tris-HCl, pH 7.6, 137mM NaCl) containing 0.1% Tween20 (TBS-T) for 1h at RT (30°C) and subsequently incubated overnight with anti-PARP antibody (cat# 9542, Cell Signaling Technology, USA) at 4°C. After 3 washes with TBS-T, the membranes were incubated with HRP conjugated anti-rabbit antibody for 1h at RT and washed thrice with TBS-T. Proteins were detected with an enhanced chemiluminescence (ECL) reagent and visualized by a chemiluminescence detector (Bio-Rad Laboratories, USA). The densitometry analysis of blots was done by using Bio-Rad Image Lab 4.0 software.

2.4.5. Antiinflammatory activity

As per protocol [20a,b], primary macrophage cells were isolated from the peritoneal cavities of mice (8-week-old female Swiss albino mice) after an intra-peritoneal injection of 1.0mL of 1% peptone (BD

Biosciences, USA) 3 days before harvesting. Mice were euthanized by cervical dislocation under ether anesthesia and peritoneal macrophages were obtained by intra-peritoneal injection of PBS (pH, 7.4). Membrane debris was removed by filtering the cell suspensions through sterile gauze. The viability of cells was determined by trypan blue exclusion and the viable macrophage cells at the concentration of 0.5×10^6 live cells/mL were used for the experimentation. Cells were grown in DMEM (Dulbecco modified Eagle medium, Sigma) supplemented with 10% FBS with 1X stabilized antibiotic-antimycotic solution (Sigma) in a CO₂ incubator at 37°C with 5% CO₂ and 90% relative humidity. The effect of diosgenin analogues **10**, **13**, **14**, and **16** was evaluated on production of pro-inflammatory cytokines in LPS induced inflammation in primary macrophage cells isolated from peritoneal cavity of Swiss albino mice.

Cells were pretreated with 1µg/mL and 10µg/mL of diosgenin analogues **10**, **13**, **14**, and **16** and standard anti-inflammatory drug, dexamethasone (Sigma Aldrich, USA) for 30 min. The cells were stimulated with lipopolysaccharide (LPS; 0.5µg/mL). After incubation with LPS for 24h, supernatants were collected and immediately frozen at -80°C. Harvested supernatants were tested for quantification of pro-inflammatory cytokines using mouse specific Enzyme Immuno Assay (EIA) Kits (BD Biosciences, USA) following the manufacturer's protocol. Briefly, the ELISA plates were coated (100µL/well) with specific mouse TNF-α and IL-6 capture antibody respectively and incubated overnight at 4°C. The plate was blocked with 200µL/well assay diluents. Culture supernatant and standard (100µL) were added into the appropriate coated wells and incubated for 2h at room temperature (20–25°C). After incubation, the plates were washed thoroughly 5 times with wash buffer. 100µL of detecting solution (detection antibody and streptavidin HRP) were added into each well. Plates were sealed and incubated for 1h at RT and then the plates were washed thoroughly 5 times with wash buffer. 100µL of tetramethylbenzidine (TMB) substrate solution were added to each well and incubated (without plate sealer) for 30 min at room temperature in the dark. 50µL of stop solution (2N H₂SO₄) was added to each well. The color density was measured at 450 and 570 nm using a microplate reader (Molecular Devices, USA). Subtract absorbance at 570 nm from absorbance 450 nm. The values of TNF-α and IL-6 were expressed as pg/mL. The % inhibition of pro-inflammatory cytokine production was calculated as follows:

$$\% \text{ inhibition} = 100 \times (\text{concentration of vehicle control} - \text{concentration of test treatment}) / \text{OD of concentration of vehicle control}, \text{ where vehicle control indicates cells treated with vehicle in LPS-induced inflammation.}$$

2.4.6. *In-vivo acute oral toxicity for safety studies*

In view of significant anti-cancer activity of compound **16** in *in-vitro* model, acute oral toxicity of the same was carried out in Swiss albino mice. Experiment was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) test guideline No 423 (1987). For this study, 30 mice (15 male and 15 female) were taken and divided into five groups comprising 3 male and 3 female mice in each group (n=6) weighing between 20-25 g. The animals were maintained at 22±5°C with humidity control and also on an automatic dark and light cycle of 12h. The animals were fed with the standard mice feed and provided *ad libitum* drinking water. Mice of Group I was kept as

control and animals of Groups II, III, IV and V were kept as experimental. The animals were acclimatized for 7 days in the experimental environment prior to the actual experimentation. Compound **16**, suspended in carboxymethylcellulose (CMC, 0.7%) was given at 5, 50, 300 and 1000mg/kg body weight to animals of Groups II, III, IV and V respectively once orally. Control animals received only vehicle. The animals were checked for mortality and any signs of ill health at hourly interval on the day of administration of compound **16** and thereafter a daily general case side clinical examination was carried out including changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, pilo-erection respiratory patterns, changes in gait, posture and response to handling were also recorded [21]. In addition to observational study, body weights were recorded and blood and serum samples were collected from all the animals on 7th day of the experiment. The samples were analysed for total RBC, WBC, differential leucocytes count, haemoglobin percentage and biochemical parameters like ALP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, serum protein and tissue protein activity. The animals were then sacrificed and were necropsed for any gross pathological changes. Weights of vital organs like liver, heart, kidney etc. were also recorded [22].

2.4.7. Statistical analysis

Statistical analysis for cytotoxicity was carried out in Microsoft Excel. Antiinflammatory results were presented as the means \pm SE and analyzed using GraphPad Prism 4. The ANOVA followed by Tukeys multiple comparison test was used to assess the statistical significance of vehicle verses treatment groups. Results are presented as the means \pm SE. Differences with a P value <0.05 were considered significant.

3. Results

3.1. Chemistry

Scheme-1 denotes the synthetic strategy of these derivatives starting with diosgenin (**1**). The 3-alcohol of diosgenin (**1**) was protected as acetyl derivative by treating it with acetic anhydride-pyridine in dry chloroform at room temperature to get diosgenin 3-acetate (**2**) in 93% yields. Derivative **2** was oxidized at 7-position by treating with Collins reagent i.e. Dipyridine-CrO₃ in dry dichloromethane to afford diosgenin 7-oxo-3-acetate (**3**) in 68% yield. The Collins reagent was prepared *in-situ* from dry pyridine and CrO₃ in dichloromethane during the reaction and subsequently substrate **2** was added. 7-Keto derivative (**3**) was converted to keto-oxime (**4**) by treating it with hydroxylamine hydrochloride in dry pyridine under reflux in 96% yield. The oxime hydroxyl was used as a handle to hook-up various ethylbromo fatty acid esters. Oxime **4** was refluxed with various ethylbromo fatty acid esters in THF-sodium hydride to get fatty acid esters of diosgenin-7-oxime (**5-10**) in quantitative yields (68-87%). All these esters were carefully hydrolysed by treating with 5% aqueous-alcoholic (1:9) KOH to give corresponding free fatty acids (**11-16**). Wittig reaction on 7-keto derivative (**3**) with various benzylic Wittig salts (Ph₃P-CH₂-Ar, 4-nitro, 3,4,5-trimethoxy, 3,4-methylenedioxy) was unsuccessful and

always yielded unexpected 3,5-dienone derivative (**17**) as an exclusive product. All these products were purified through column chromatography and confirmed by spectroscopy [Supporting information].

[Scheme 1]

Compound **17** was obtained as white amorphous powder. Its structure was established by spectroscopy. Its molecular formulae $C_{27}H_{38}O_3$ was determined by its ^{13}C NMR and ESI-HR-MS at m/z 411.2872 $[M+H]^+$ (Calcd. 411.2899). In 1H NMR acetyl protons were absent which was further confirmed in ^{13}C NMR (δ 21.6 and 170.6 both absent). ^{13}C NMR showed total 27 distinct carbons (four CH_3 , eight CH_2 , ten CH and five quaternary) indicating two less carbons as compared to substrate **3** which was for 3-acetyl group. There were six resonances at δ 109.51 (C22), 124.32 (C6), 128.05 (C4), 137.13 (C3), 161.59 (C5) and 202.14 (C7) in the transformed product **17**. Absence of acetyl group and introduction of one more double bond ($\Delta^{3,4}$) showed possibility of dehydroacetoxylation of 3-acetoxy group as acetic acid. Rest of the carbon resonances were similar to substrate **3** exhibiting intact rings C, D, E and F. With all these observations the structure of transformed product **17** was confirmed as (22 β ,25R)-7-oxo-spirosta-3,5-diene.

The purity of compounds **9**, **19**, **11**, **13**, **14** and **16** was determined following high-low chromatographic approach by changing hydrophobic interactions without any acid additives unless otherwise mentioned. The purity of the compounds was 96.0-99.4%. As expected, mobile phase-I (70% acetonitrile and water) produced more symmetrical peak shapes than mobile phase composition –II and earlier retention times (*mobile phase-I*: 9.937, 3.261, 3.268, 9.067, 6.662 and 5.811min; *mobile phase-II*: 12.208, 4.935, 4.971, 8.847, 6.730 and 6.607 min) for all the compounds analyzed except compound **16**. Broad peak shape of compound **16** was observed when chromatographed with mobile composition-II due structural characteristic (presence of heptanoid free acid chain) which became symmetrical on introducing acetic acid (1%, v/v) as ion-pairing reagent by reducing charge-charge interaction.

3.2. Pharmacology

3.2.1. Cytotoxicity

All the semi-synthetic analogues were evaluated for anticancer activity against a panel of human cancer cell lines viz. FaDu (hypopharyngeal carcinoma), A549 (Lung carcinoma), DLD1 (colorectal adenocarcinoma), MCF-7 (Breast adenocarcinoma), DU145 (prostate carcinoma) and PC3 (Prostate carcinoma) (Table 1). Out of thirteen analogues only eight analogues exhibited significant cytotoxicity ($IC_{50} < 50 \mu M$). Rest of the analogues was declared as inactive. However, analogues **13** and **16** exhibited good activity. Most of the fatty acids derivatives of diosgenin were active. Fatty acid ester derivatives with six (**9**) and seven (**10**) carbon chain were active. Diosgenin 7-ketoxime analogue with longest fatty acid chain was the best analogue of the series. Analogue **16** was the best representative from the series exhibiting potent anticancer activity ($IC_{50}=12.8 \mu M$) against prostate cancer cell line DU-145.

[Table 1]

3.2.2. Cell cycle analysis

Cell cycle is an important phenomenon in cells for their proliferation and multiplication. It is controlled by a complex series of signaling pathways by which cell grows, replicates its DNA and divides. In cell cycle analysis, diosgenin analogue **16** significantly induced S phase arrest in DU-145 prostate cancer cells at 13 μ M concentration. In 24h incubation S phase arrest was quite significant but there was no such effect at 48h incubation. (Figure 2).

[Figure 2]

3.2.3. Western blot experiment

In the present study, we found cleavage of PARP in DU-145 cells at 24h exposure to the diosgenin analogue **16** at 13 μ M concentration implying activation of caspases by the molecule to trigger apoptosis (Figure 3).

[Figure 3]

3.2.4. Antiinflammatory activity of diosgenin derivatives

Cancer and inflammation are associated with each other. The antiinflammatory effect of these cytotoxic compounds was assessed against lipopolysaccharide (LPS) induced proinflammatory cytokines (TNF- α and IL-6) (Table 2). All the four diosgenin derivatives **10**, **13**, **14** and **16** exhibited moderate level of antiinflammatory activity through inhibition of TNF- α (31.8-47.7%) at 1 μ g/mL and 10 μ g/mL doses. However, inhibition of IL-6 was very low (13.8-23.3%) as compared to diosgenin and dexamethasone. Anticancer agent with antiinflammatory property is rare and more desirable.

[Table 2]

3.2.5. Safety studies

There were no observational changes, morbidity and mortality during the experimental period in all the group of experimental animals up to the tested dose levels of 1000mg/kg. Blood and serum samples did not show any significant changes in the parameters studied like haemoglobin level, RBC count, WBC count, differential leucocytes count, SGPT, ALP, creatinine, triglycerides, cholesterol, albumin, serum protein (Table 3 & Fig. 5) except serum albumin and SGOT levels. Serum albumin level was found to be significantly higher (Table 4) in the group of the animals treated with the test compound at 1000mg/kg body weight compare to the control while SGOT level was found to be significantly higher (Table 3) in the group of animals treated with the test compound at 1000mg/kg body weight compare to control and all other treatment groups. Animals on gross pathological study did not show any change in any of the organs studied including their absolute and relative weight (Figure 4). Since, rise in serum albumin level indicates damage of liver function and burden on vital function of kidney and immune system and additional significant rise in the level of SGOT indicate that the test compound **16** found to be toxic at 1000mg/kg body weight. Therefore, the experiment showed that compound **16** is well tolerated by the Swiss albino mice up to the dose level of 300mg/kg body weight as a single acute oral

dose. However, it is advisable to conduct sub-acute and or chronic experiment with the test drug to observe for any adverse effect on continuous exposure of compound **16** in view of significant increase in serum albumin and SGOT level in animals treated with the test drug at 1000mg/kg.

[Table 3]

[Figure 4] [Figure 5]

4. Discussion

Both dioscin and diosgenin exhibit cancer chemopreventive and cytotoxic effects [23a,b]. Diosgenin suppresses fatty acid synthase expression through modulating Akt, mTOR and JNK phosphorylation [24]. While, dioscin induces apoptosis by the activation of p38 MAPK and JNK through the caspase dependent mitochondrial death pathway in HL-60 cells [25]. The long chain fatty acid/ester analogues of diosgenin have shown moderate cytotoxic activity against DU-145 prostate cancer cell line. Compound **16** exhibited good anticancer activity ($IC_{50}=12\mu M$).

Prostate cancer is the second most frequently diagnosed cancer in men accounting about 15% of all male cancers [26]. However, its prevalence is higher and prognoses is poorer in developed countries than the rest of the world. A complete understanding of the causes of prostate cancer remains unclear [27]. The treatment includes surgery, radiation therapy, brachytherapy, high intensity focused ultrasound, chemotherapy, hormonal therapy or some combinations [28a,b]. But, most of the hormone dependent cancers has become resistant to treatment after one to three years and regain growth against hormone therapy [29]. There is still a need for suitable anti-prostate cancer drug.

The cytotoxic effect of compound **16** was found to be through activation of caspase pathway. In our western blot experiments compound **16** cleaved PARP (Poly ADP-ribose polymerase), a family of cysteine-aspartic protease proteins involved in a number of cellular processes like DNA repair and programmed cell death. Caspase family plays an important role in the activation of apoptosis. Caspase-3 is a critical promoter of apoptosis. It is responsible for proteolytic degradation of PARP which facilitates cellular disassembly serving as a marker cells leading to apoptosis [30]. Both caspase-3 and caspase-9 are crucial to initiate caspase cascade leading to apoptosis. Caspase-9 is an initiator caspase while caspase-3 is an effector (executioner) caspase [31]. PARP is involved in DNA repair when cell experiences environmental stress [32a]. During apoptosis, PARP is cleaved by caspases and inactivated [32b,c]. Hence, cleavage of PARP is a remarkable marker for detection of apoptosis. In the present study, we found cleavage of PARP in DU-145 cells at 24h exposure to the compound **16** implying activation of caspases by the molecule to trigger apoptosis. In some of the cancer cells diosgenin induces apoptosis through caspase pathway [33].

Cell cycle regulation ensures the precision in genomic replication and cell division [34]. The two major checkpoints G_1/S and G_2/M transitions allow the cells to control any modification in DNA content. 'Cell cycle arrest' is a stopping point in the cell cycle, where it is no longer involved in the processes related to duplication and division. Diosgenin analogue **16** caused S phase arrest in 24h in DU-145 cells and induced apoptosis. Induction of cell cycle arrest in cancer cell lines constitutes one of the most prevalent strategies to stop or limit cancer spreading [35].

Cancer and inflammation have been linked with each other [36a,b]. Sometimes prolonged inflammation becomes cause to cancer. Inflammation is associated to cancer even after development of cancer. Role of NF- κ B and many other inflammatory mediators like cytokines (TNF α etc.) in tumor promotion have been linked [37]. These pro-inflammatory factors are over-expressed during the progression of cancer. Diosgenin analogues **14** and **16** inhibited TNF- α and IL-6 expressions moderately at 1 μ g/mL and 10 μ g/mL concentrations. This additional property is very important and desirable in anticancer agents. Diosgenin and its analogues have been reported to possess antiinflammatory property previously [38].

In acute oral toxicity compound **16** was found to be safe up to 300mg/kg oral dose. But at a higher dose of 1000mg/kg, there were some indications of liver damage. There was concentration dependent liver toxicity only in two parameters of liver i.e. SGOT and albumin, rest other parameters were normal. In case of anticancer drug through oral administration, it is possible. Soft drug design approach should be applied to tackle this problem. However, therapeutic index will be important to monitor the dose and route of administration of this compound.

4. Conclusion

Present study has provided several novel analogues of diosgenin at C7 position. Two of the analogues possessed significant anticancer and antiinflammatory activities simultaneously, which is rare and desirable. Analogue **16**, the best representative of the series, exerted cell cycle arrest at S phase and induced apoptosis. The mechanism of antiproliferative action of compound **16** is through caspase dependent apoptosis pathway. Compound **16** is a safe anticancer derivative of diosgenin. The lead obtained in this study can further be optimized for better activity in future.

Supporting Information

Spectral data of compounds are available on online version.

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Figures, Tables and Scheme

Figure 1: Various bioactive diosgenin related compounds and present prototype.

Figure 2: Cell cycle analysis of analogue **16** in DU-145 cells

Figure 3: Cleavage of PARP, a caspase target by compound **16** in DU-145 cell lines.

Figure 4A. *In-silico* molecular docking studies elucidating the possible mechanisms of diosgenin (**1**), its analogues **14** and **16**, and positive control doxorubicin with Caspase-9 (PDB: **1NW9**). The docking studies were carried out using 'AutoDock Vina'.

Figure 4B. *In-silico* molecular docking studies elucidating the possible mechanisms of diosgenin (**1**), its analogues **14** and **16**, and positive control doxorubicin with Caspase-3: (PDB: **3KJF**). The docking studies were carried out using 'AutoDock Vina'.

Figure 5. Effect of compound **16** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on absolute and relative organ weight in Swiss albino mice (n=6, Non significant changes were found compared to control).

Figure 6. Effect of compound **16** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg body weight on differential leucocytes counts in Swiss albino mice (n=6, Non significant changes were found compared to control).

Table 1. Cytotoxicity activity of diosgenin analogues against human cancer cell line by Sulphorhodamine assay (incubation period=48h);

Table 2: Effect of Diosgenin analogues on production of LPS-induced pro-inflammatory cytokines in primary macrophage cells

Table 3. Details about docking studies carried out for finding of molecular interactions of compounds under study

Table 4. Effect of compound **16** as a single acute oral dose at 5, 50, 300 and 1000 mg/kg on body weight, haematological and serum biochemical parameters in Swiss albino mice (Mean±SE; n=6; * P<0.05 compared to control; a P<0.05 compared to control, 5, 50, 300, 1000 mg/kg).

Scheme 1: Reagents and conditions: (a) Ac₂O, dry pyridine, dry CHCl₃, RT, 2h, 91%; (b) Dry DCM, dry Pyridine, CrO₃, 4h, 68%; (c) dry pyridine, NH₂OH-HCl, reflux, 2h, 96%; (d) dry THF, NaH, ethylbromo ester, RT, 0.5h-2h, 70-92%; (e) 1.2% KOH in water:MeOH (1:9), RT, 1-2h, 84-91%; (f) dry toluene, PPh₃.CH₂-Ar, NaH, reflux, 2-4h, 84-91%.

Figure 1

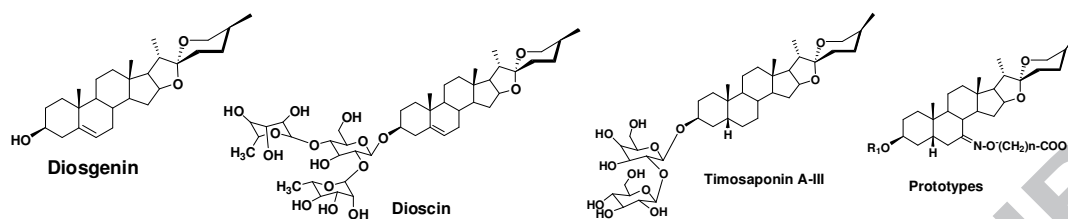


Figure 2

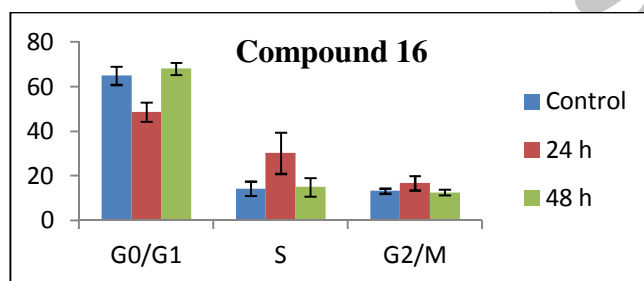


Figure 3

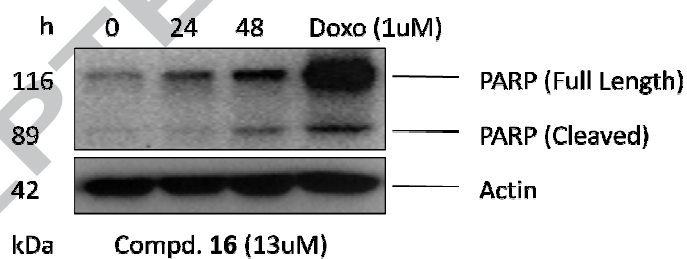
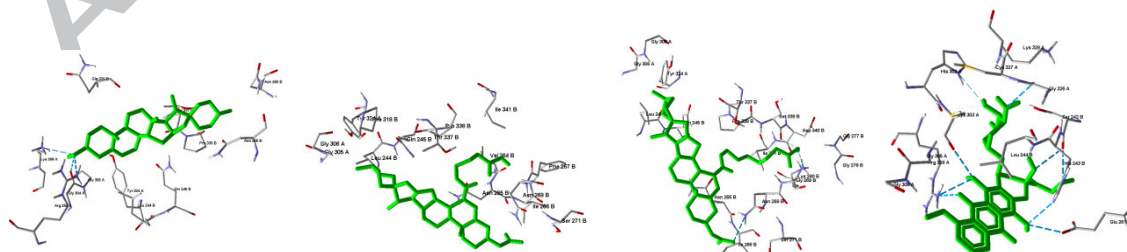


Figure 4A



Diosgenin

Analogue 14

Analogue 16

Doxorubicin

Figure 4B:

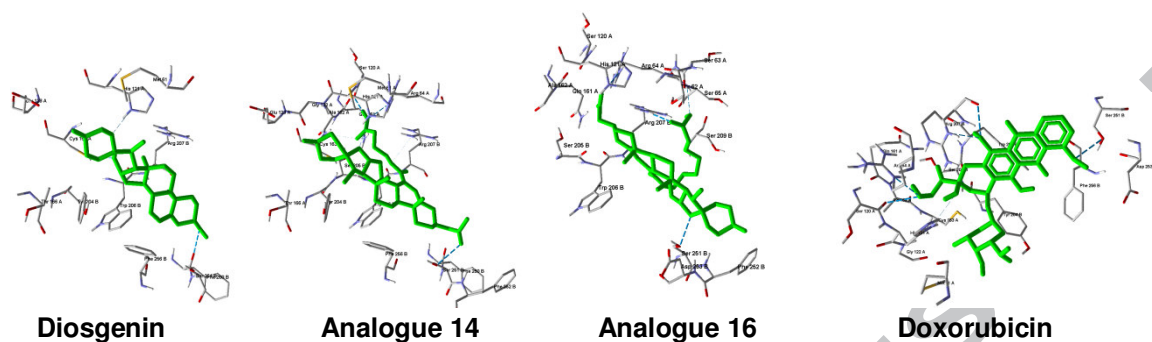


Figure 5

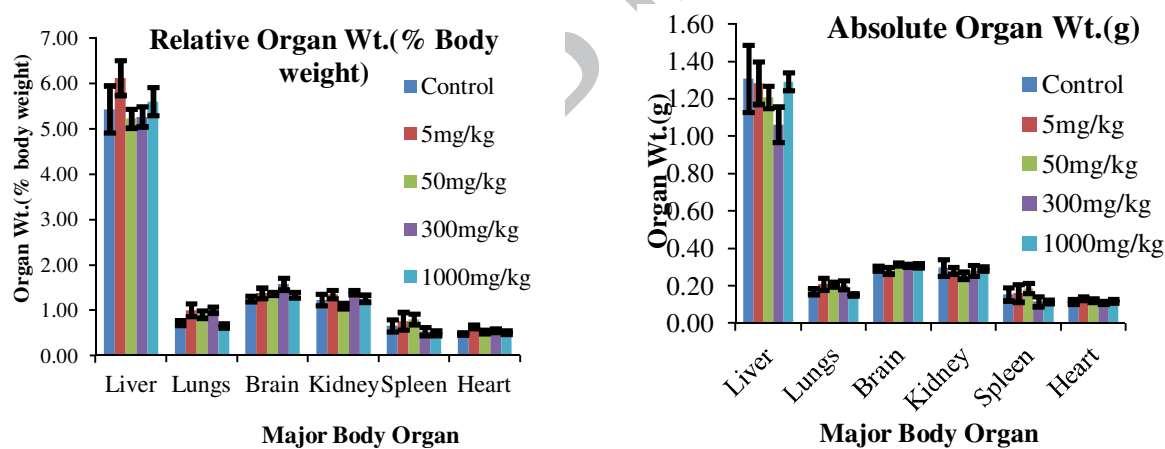


Figure 6.

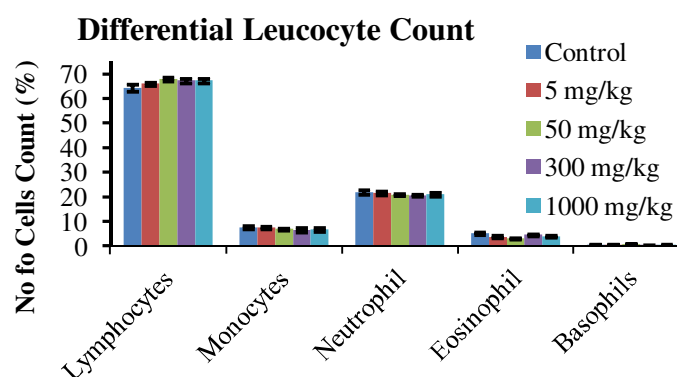


Table 1:

Compound Name	IC ₅₀ (μ M) (Mean \pm SE) ^a					
	FaDu	A549	DLD1	MCF-7	DU145	PC3
9	33.30 \pm 5.89	>50	35.49 \pm 2.66	35.10 \pm 3.54	31.52 \pm 2.56	---
10	26.95 \pm 7.19	21.92 \pm 4.18	40.20 \pm 2.32	29.43 \pm 5.63	35.43 \pm 0.48	---
11	31.64 \pm 6.02	29.04 \pm 3.45	31.22 \pm 160	33.34 \pm 0.98	33.03 \pm 0.60	---
13	21.95 \pm 3.06	26.20 \pm 3.99	22.70 \pm 3.13	22.73 \pm 4.59	21.19 \pm 2.14	---
14	16.56\pm1.57	17.05\pm3.61	16.01\pm1.88	15.59\pm1.89	14.67\pm1.26	21.85\pm0.53
16	15.21\pm0.90	15.77\pm2.22	14.55\pm0.64	15.77\pm1.98	12.82\pm1.26	25.39\pm1.70
Doxorubicin	<1.25	<1.25	2.19 \pm 0.34	<1.25	<1.25	<1.25
Podophyllotoxin	ND	<1.25	0.104 \pm 0.013	<1.25	<1.25	<1.25
Tamoxifen	8.29 \pm	15.68 \pm	16.34 \pm	10.07 \pm 0.46	19.82 \pm	---

a=mean of three independent experiments (n=3); ^aIC₅₀>50 μ M inactive.

Table 2:

Compound	LPS (0.5 μ g/mL)	Dose (μ g/mL)	TNF- α (pg/mL)	TNF- α % inhibition	IL-6 (pg/mL)	IL-6 % Inhibition
Normal	-	-	24.66 \pm 0.72	NA	288.33 \pm 85.11	NA
Vehicle	0.5 μ g/mL	-	153 \pm 6.17 [#]	00	2733 \pm 29.40 [#]	00
10	0.5 μ g/mL	1	104.33 \pm 7.21 ^{***}	31.80 \pm 4.71	2354 \pm 61.69 ^{**}	13.86 \pm 2.25
		10	94.16 \pm 2.42 ^{***}	38.45 \pm 1.58	2295 \pm 55.01 ^{***}	16.02 \pm 2.01
13	0.5 μ g/mL	1	101.33 \pm 2.20 ^{***}	33.76 \pm 1.44	2353.66 \pm 20.40 ^{**}	13.02 \pm 0.74
		10	98.16 \pm 4.47 ^{***}	35.83 \pm 2.92	2261.66 \pm 84.84 ^{***}	17.24 \pm 3.10
14	0.5 μ g/mL	1	100.66 \pm 9.95 ^{***}	34.20\pm6.50	2339 \pm 40.46 ^{**}	14.41 \pm 1.48
		10	80 \pm 4.19 ^{***}	47.71\pm2.74	2095.33 \pm 88.64 ^{***}	23.33\pm3.24
16	0.5 μ g/mL	1	97 \pm 7.21 ^{***}	36.60\pm4.71	2251 \pm 78.83 ^{***}	17.63 \pm 2.88
		10	89.16 \pm 11.98 ^{***}	41.72\pm7.83	2193.66 \pm 45.60 ^{***}	19.73\pm1.66
Diosgenin	0.5 μ g/mL	1	117.50 \pm 5.77 [*]	23.20 \pm 3.77	2397.66 \pm 75.51 [*]	12.26 \pm 2.76

		10	95.16±5.08 ^{***}	37.79±3.32	2048±36.75 ^{***}	25.06±1.34
Dexamethasone	0.5µg/mL	1	59.5±7.48 ^{***}	61.11±4.89	925.66±75.13 ^{***}	66.13±2.74
		10	35.83±9.53 ^{***}	76.57±6.23	694 ±86.49 ^{***}	74.60±3.16

Normal vs Vehicle; [#] P<0.05 ; Vehicle vs Treatment; ^{ns} -not significant, P<0.05; n=3

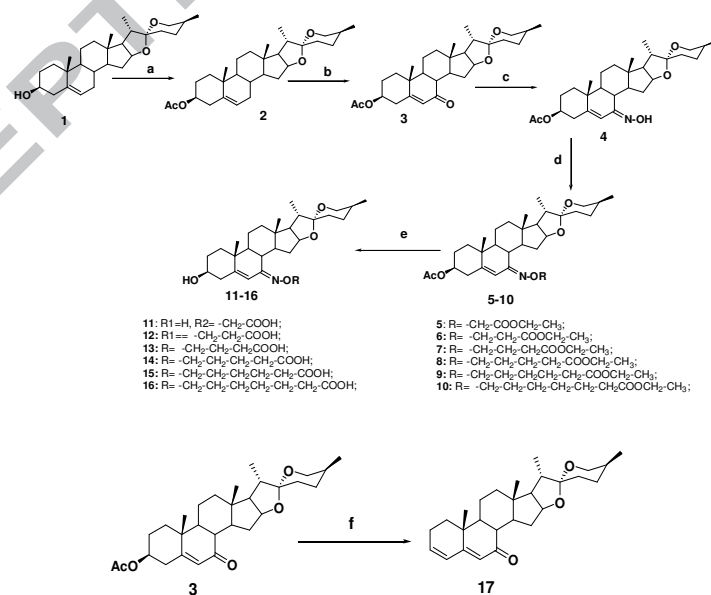
Table 3.

<i>Compd code</i>	<i>Caspase-3 (PDB entry 3kjf)</i>		<i>Caspase-9 (PDB entry 1nw9)</i>	
	<i>Binding affinity (Kcal/mol)</i>	<i>Binding pocket amino acids</i>	<i>Binding affinity (kcal/mol)</i>	<i>Binding pocket amino acids</i>
14	-10.2	ALA[A]-162, ARG[A]-64, CYS[A]-163, GLN[A]-161, GLU[A]-123, GLY[A]-122, GLY[A]-165, HIS[A]-121, MET[A]-61, PHE[A]-128, SER[A]-120, THR[A]-62, THR[A]-166, ARG[B]-207, ASP[B]-253, PHE[B]-250, PHE[B]-252, PHE[B]-256, SER[B]-205, SER[B]-251, TRP[B]-206, TYR[B]-204	-7.4	GLY[A]-304, GLY[A]-305, GLY[A]-306, TYR[A]-324, ASN[B]-265, ASN[B]-268, GLN[B]-245, GLN[B]-320, GLU[B]-261, ILE[B]-266, ILE[B]-341, LEU[B]-217, LEU[B]-244, LYS[B]-262, LYS[B]-280, PHE[B]-246, PHE[B]-267, PHE[B]-319, PRO[B]-318, PRO[B]-338, SER[B]-271, SER[B]-339, THR[B]-337, VAL[B]-264
16	-8.3	ALA[A]-162, ARG[A]-64, CYS[A]-163, GLN[A]-161, HIS[A]-121, SER[A]-63, SER[A]-65, SER[A]-120, THR[A]-62, ARG[B]-207, ASN[B]-208, ASP[B]-253, PHE[B]-250, PHE[B]-252, PHE[B]-256, SER[B]-205, SER[B]-209, SER[B]-251, TRP[B]-206	-7.7	GLY[A]-304, GLY[A]-305, GLY[A]-306, TYR[A]-324, ASN[B]-265, ASN[B]-268, ASP[B]-340, GLN[B]-245, GLN[B]-320, GLU[B]-261, GLY[B]-269, GLY[B]-276, GLY[B]-277, ILE[B]-266, ILE[B]-341, LEU[B]-217, LEU[B]-244, LEU[B]-275, LYS[B]-280, PHE[B]-246, PHE[B]-267, PHE[B]-319, PRO[B]-318, PRO[B]-338, SER[B]-271, SER[B]-339, THR[B]-337, VAL[B]-264
Diosgenin	-8.4	CYS[A]-163, GLU[A]-123, GLY[A]-122, GLY[A]-165, HIS[A]-121, MET[A]-61, PHE[A]-128, THR[A]-62, THR[A]-166, ARG[B]-207, PHE[B]-250, PHE[B]-256, SER[B]-205, SER[B]-251, TRP[B]-206, TYR[B]-204	-8.5	ARG[A]-258, GLY[A]-304, GLY[A]-305, GLY[A]-306, LYS[A]-299, TYR[A]-324, ASN[B]-265, ASN[B]-268, GLN[B]-245, GLN[B]-320, GLU[B]-261, GLY[B]-269, ILE[B]-341, LEU[B]-244, PHE[B]-246, PHE[B]-267, PHE[B]-319, PRO[B]-318, PRO[B]-338, SER[B]-339, THR[B]-337
Doxorubicin	-8.2	ALA[A]-162, ARG[A]-64, CYS[A]-163, GLN[A]-161, GLY[A]-122, HIS[A]-121, MET[A]-61, SER[A]-120, ARG[B]-207, ASN[B]-208, ASP[B]-253, PHE[B]-250, PHE[B]-252, PHE[B]-256, SER[B]-205, SER[B]-251, TRP[B]-206, TYR[B]-204	-8.2	ARG[A]-258, CYS[A]-303, CYS[A]-327, GLY[A]-304, GLY[A]-305, GLY[A]-306, GLY[A]-326, HIS[A]-302, LEU[A]-256, LYS[A]-299, LYS[A]-328, GLU[B]-261, HIS[B]-243, LEU[B]-244, SER[B]-242

Table 4:

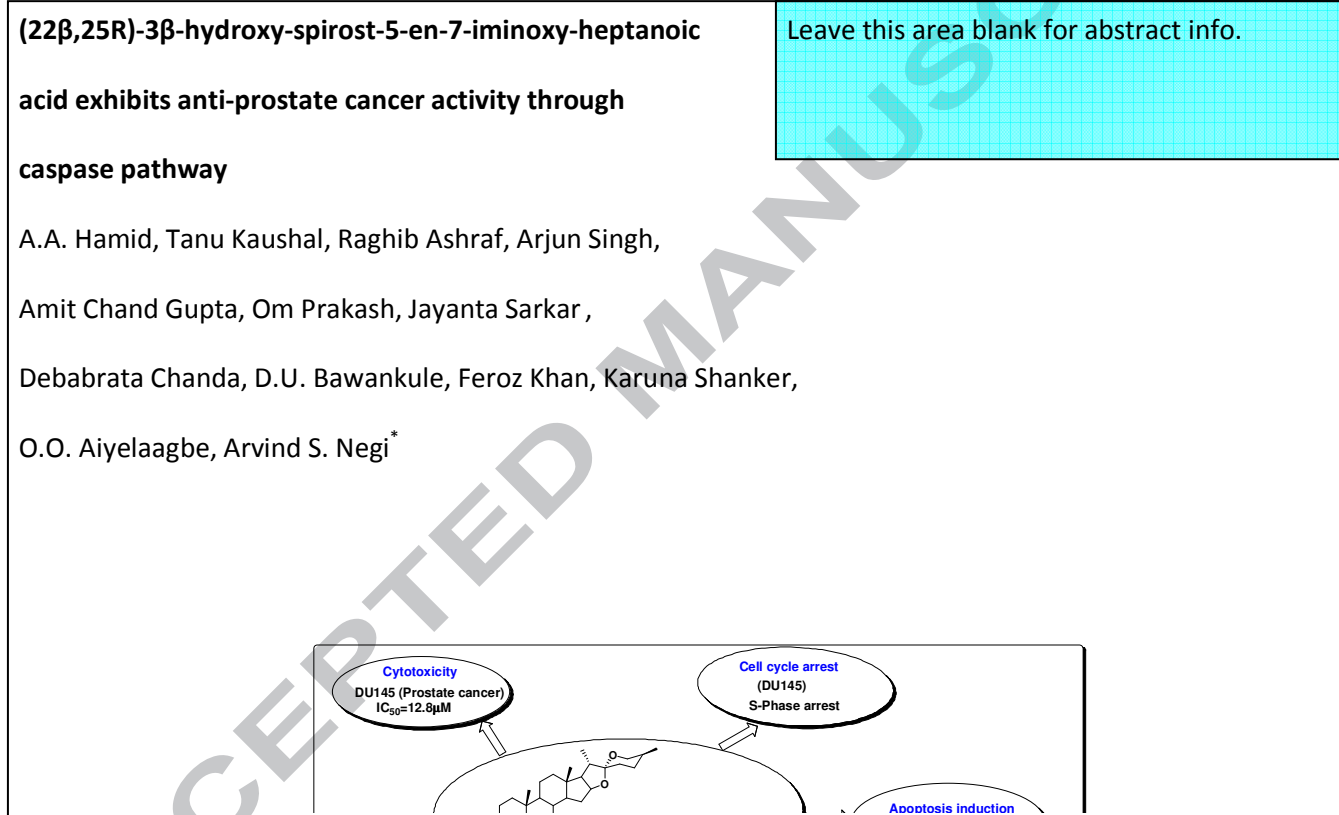
Parameters	Dose of compound 16 at mg/kg body weight as a single oral dose				
	Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
Body wt. 7 th day (g)	24.18±1.82	21.35±1.85	23.0±0.74	22.41±1.53	23.26±0.91
Haemoglobin (g/dL)	11.19±.39	12.07±0.50	12.51±0.63	11.80±0.47	11.36±0.41
RBC (millions/mm ³)	6.29±0.26	6.17±0.29	6.28±0.03	5.93±0.32	5.54±0.34
WBC (thousands/mm ³)	11.27±0.61	12.49±1.07	10.84±0.35	11.32±0.64	11.68±0.39
ALP (U/L)	264.1±20.6	167.3±16.5	191.6±27.6	224.0±16.70	234.3±24.5
SGOT (U/L)	29.77±2.07	33.79±2.19	31.86±2.13	33.05±2.35	43.80±1.19 ^a
SGPT (U/L)	22.41±1.06	24.24±0.86	25.93±1.30	24.47±1.45	25.21±1.32
Albumin (g/dL)	5.03±0.25	6.45±1.17	7.20±0.85	6.47±0.18	8.48±1.07*
Creatinine (mg/dL)	0.51±0.04	0.63±0.10	0.55±0.13	0.74±0.08	0.50±0.05
Triglycerides (mg/dL)	123.2±11.56	139.5±8.99	145.8±18.47	135.2±16.08	133.1±13.45
Serum Protein (mg/ml)	0.95±0.02	0.97±0.08	1.00±0.07	0.90±0.05	0.85±0.05
Cholesterol (mg/dL)	277.80±18.9	295.97±21.7	270.90±23.4	337.96±14.7	281.37±18.6
Bilirubin (mg/dL)	0.06±0.01	0.14±0.07	0.09±0.01	0.08±0.02	0.12±0.04

Scheme 1:



Graphical Abstract

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Highlights

- * Diverse long chain fatty acid/esters of diosgenin-7-ketoxime have been prepared.
- * Analogue **16**, cytotoxicity $IC_{50}=12\mu M$ prostate cancer, cell cycle arrest at S phase.
- * Induces apoptosis through caspase pathway, shows affinity to caspase-3/9.
- * Simultaneously antiinflammatory property by inhibition of TNF- α and IL-6.
- * Safe up to 1000mg/kg dose in Swiss-albino mice.
- * Simultaneous, antiinflammatory property in an anticancer molecule is favourable.