



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



A.A. Hamid<sup>a,c</sup>, Tanu Kaushal<sup>a</sup>, Raghbir Ashraf<sup>b</sup>, Arjun Singh<sup>a</sup>, Amit Chand Gupta<sup>a</sup>, Om Prakash<sup>a</sup>, Jayanta Sarkar<sup>b</sup>, Debabrata Chanda<sup>a</sup>, D.U. Bawankule<sup>a</sup>, Feroz Khan<sup>a</sup>, Karuna Shanker<sup>a</sup>, O.O. Aiyelaagbe<sup>d</sup>, Arvind S. Negi<sup>a,\*</sup>

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

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

<sup>c</sup> Department of Chemistry, University of Ilorin, Ilorin, Nigeria

<sup>d</sup> Organic Chemistry Unit, Department of Chemistry, University of Ibadan, Ibadan, Nigeria

### ARTICLE INFO

#### Article history:

Received 16 September 2016

Received in revised form 16 December 2016

Accepted 12 January 2017

Available online 28 January 2017

#### Keywords:

Diosgenin  
Oxime derivatives  
Anticancer  
Caspase pathway  
Antiinflammatory  
Acute oral toxicity

### 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 IC<sub>50</sub> ranging from 12 to 35  $\mu$ 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- $\alpha$  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 300 mg/kg dose. The anticancer and antiinflammatory properties of compound **16** are important and can further be optimized for a better anti-prostate cancer candidate.

© 2017 Elsevier Inc. All rights reserved.

### 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, antiprogesterins, 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].

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

\* Corresponding author.

E-mail address: [arvindcimap@rediffmail.com](mailto:arvindcimap@rediffmail.com) (A.S. Negi).

<sup>1</sup> CIMAP Communication No. CIMAP/PUB/2016/45.

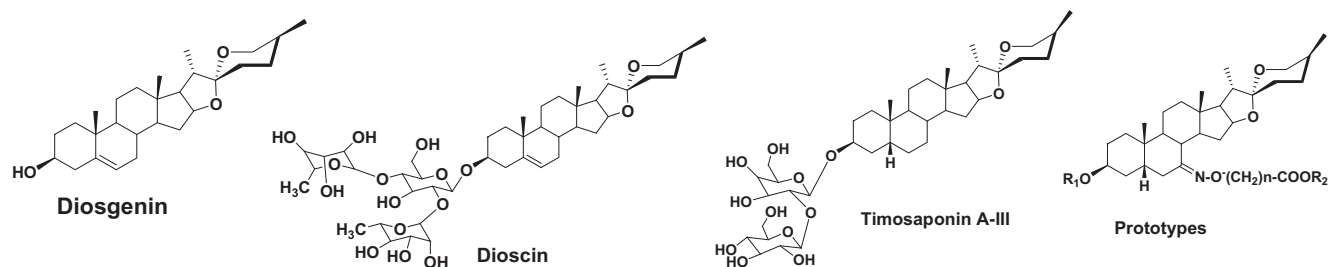


Fig. 1. Various bioactive diosgenin related compounds and present prototype.

effect of both the analogues was also checked on production of LPS induced proinflammatory cytokines (TNF- $\alpha$  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.

## 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 (254 nm and 365 nm), 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 (300 MHz/500 MHz) spectrometer with TMS as internal standard ( $\delta$ , 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 $\beta$ ,25R)-spirost-5-en-3 $\beta$ -yl-3-acetate (**2**)

Diosgenin (1 g, 2.4 mmol) was taken in dry chloroform (10 mL). To this stirred solution, DMAP (50 mg) and acetic anhydride (1 mL, 10.5 mmol) were added and further stirred for 2 h at RT. The reaction mixture was diluted with water and extracted with chloroform, washed with dil. HCl (5%, 10 mL) 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 ( $CDCl_3$ ),  $\delta$  0.77 (s, 3H, 18- $CH_3$ ), 0.96 (d, 3H, 27- $CH_3$ ,  $J$  = 6.9 Hz), 1.02 (s, 3H, 19- $CH_3$ ), 1.11–2.31 (m, 25H, rest of the 1  $\times$   $CH_3$ , 8  $\times$   $CH_2$  and 6  $\times$  CH 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);  $\delta$  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  $C_{29}H_{44}O_4$ : 457.3 [M+H] $^+$ , 479.3 [M+Na] $^+$ , 495.4 [M+K] $^+$ . IR (KBr,  $cm^{-1}$ ): 2907, 1724, 1451, 1231.

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

A mixture of dry methylene chloride (25 mL) and dry pyridine (10 mL) was stirred with cooling (5–10 °C). To this crushed chromium trioxide (1 g) was added and further stirred for 10 min. To this reaction mixture diosgenin 3-acetate (**2**, 500 mg, 1.09 mmol) was added as a solution in methylene chloride (3–5 mL) drop-wise. It was stirred for 30 min in ice-bath and then at room temperature for 4 h. On completion methanol (2 mL) 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 = 351 g (68%), mp = 179–182 °C;  $^1H$  NMR ( $CDCl_3$ ),  $\delta$  0.78 (s, 3H, 18- $CH_3$ ), 0.97 (d, 3H, 27- $CH_3$ ,  $J$  = 6.6 Hz), 1.14 (s, 3H, 19- $CH_3$ ), 1.16–2.84 (m, 23H, rest of the 1 $\times$  $CH_3$ , 7 $\times$  $CH_2$  and 6 $\times$ CH 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);  $\delta$  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  $C_{29}H_{42}O_5$ : 471.4 [M+H] $^+$ , 493.4 [M+Na] $^+$ , 509.3 [M+K] $^+$ . IR (KBr,  $cm^{-1}$ ): 2907, 1724, 1451, 1231.

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

Diosgenin 7-ketone (**3**, 500 mg, 1.06 mmol) was taken in dry pyridine (6 mL). To this hydroxylamine hydrochloride (150 mg, 2.16 mmol) was added and refluxed for 2 h. On completion, water was added to it, acidified with HCl (5%, 10 mL), 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 = 494 mg (96%), oil;  $^1H$  NMR ( $CDCl_3$ ),  $\delta$  0.77 (s, 3H, 18- $CH_3$ ), 0.97 (d, 3H, 27- $CH_3$ ,  $J$  = 6.6 Hz), 1.11 (s, 3H, 19- $CH_3$ ), 1.20–2.41 (m, 21H, rest of the 1  $\times$   $CH_3$ , 7  $\times$   $CH_2$  and 6  $\times$  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).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz);  $\delta$  14.97 (C21), 17.08 (C18), 17.46 (C27), 18.28 (C19), 20.88 (C11), 21.67 (C3-acetate  $\text{CH}_3$ ), 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)

**2.2.4.1. Synthesis of (22 $\beta$ ,25R)-3 $\beta$ -acetoxy-spirost-5-en-7-iminoxy-ethylacetate (5).** Diosgenin 7-keto oxime (**4**, 200 mg, 0.41 mmol) was taken in dry acetone (15 mL) and anhydrous potassium carbonate (1 g). To this ethyl iodoacetate (0.1 mL, 181 mg, 0.84 mmol) was added and the reaction mixture was refluxed for 4 h. 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 **5** was obtained at 6% ethyl acetate-hexane as white amorphous solid.

**5:** Yield = 210 mg (89%), mp = 146–148 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  0.77 (s, 3H, 18- $\text{CH}_3$ ), 0.98 (d, 3H, 27- $\text{CH}_3$ ,  $J = 6.9$  Hz), 1.12 (s, 3H, 19- $\text{CH}_3$ ), 1.33 (t, 3H,  $\text{CH}_3$ , ethyl,  $J = 8.0$  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,  $\text{CH}_3\text{COO}$ , 3-acetate), 3.42 (m, 2H, 26- $\text{CH}_2$ ), 4.20 (q, 2H,  $\text{OCH}_2$ , ethyl,  $J = 7.1$  Hz) 4.54 (s, 2H,  $\text{OCH}_2$ ), 4.56 (m, 2H, 3-CH & 16-CH), 6.54 (s, 1H, 6-CH).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz);  $\delta$  14.65 (C21), 15.05 ( $\text{CH}_3$ , ethyl), 16.99 (C18), 17.51 (C27), 18.28 (C19), 20.94 (C11), 21.68 (C3-acetate  $\text{CH}_3$ ), 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 ( $\text{CH}_2$ , ethyl), (C26), 71.01 (2'- $\text{CH}_2$ ), 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,  $\text{cm}^{-1}$ ): 2947, 1737, 1638, 1451, 1241.

## 2.2.5. (22 $\beta$ ,25R)-3 $\beta$ -acetoxy-spirost-5-en-7-iminoxy-ethylpropionate (6)

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

**6:** Yield = 198 mg (82%), mp = 141–143 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  0.75 (s, 3H, 18- $\text{CH}_3$ ), 0.95 (d, 3H, 27- $\text{CH}_3$ ,  $J = 6.9$  Hz), 1.13 (s, 3H, 19- $\text{CH}_3$ ), 1.30 (t, 3H,  $\text{CH}_3$ , ethyl ester,  $J = 7.8$  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,  $\text{CH}_3\text{COO}$ , 3-acetate), 3.37–3.42 (m, 2H, 26- $\text{CH}_2$ ), 4.15 (q, 2H,  $\text{OCH}_2$ , ethyl ester,  $J = 4.7$  Hz) 4.19 (m, 2H, -N- $\text{OCH}_2$ ), 4.64 (m, 2H, 3-CH & 16-CH), 6.52 (s, 1H, 6-CH).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz);  $\delta$  14.69 (C21), 15.04 ( $\text{CH}_3$ , ethyl), 16.95 (C18), 17.50 (C27), 18.23 (C19), 20.91 (C11), 21.64 (C3-acetate  $\text{CH}_3$ ), 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 ( $\text{CH}_2$ , 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,  $\text{cm}^{-1}$ ): 2948, 1738, 1638, 1458, 1241.

## 2.2.6. (22 $\beta$ ,25R)-3 $\beta$ -acetoxy-spirost-5-en-7-iminoxy-ethylbutyrate (7)

**7:** Yield = 205 mg (83%), mp = 145–148 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  0.77 (s, 3H, 18- $\text{CH}_3$ ), 0.97 (d, 3H, 27- $\text{CH}_3$ ,  $J = 6.9$  Hz), 1.12 (s, 3H,

19- $\text{CH}_3$ ), 1.32 (bs, 3H,  $\text{CH}_3$ , 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,  $\text{CH}_3\text{COO}$ , Acetate), 3.46 (m, 2H, 26- $\text{CH}_2$ ), 4.12 (q, 2H,  $\text{OCH}_2$ , ethyl,  $J = 7.1$  Hz) 4.05 (t, 2H, -N- $\text{OCH}_2$ ,  $J = 7.2$  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,  $\text{cm}^{-1}$ ): 2928, 1737, 1637, 1459, 1241.

## 2.2.7. (22 $\beta$ ,25R)-3 $\beta$ -acetoxy-spirost-5-en-7-iminoxy-ethylpentanoate (8)

**8:** Yield = 176 mg (70%), mp = 199–202 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  0.76 (s, 3H, 18- $\text{CH}_3$ ), 0.97 (d, 3H, 27- $\text{CH}_3$ ,  $J = 6.9$  Hz), 1.19 (s, 3H, 19- $\text{CH}_3$ ), 1.23 (t, 3H,  $\text{CH}_3$ , ethyl ester,  $J = 7.1$  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,  $\text{CH}_3\text{COO}$ , 3-acetate), 3.44 (m, 2H, 26- $\text{CH}_2$ ), 4.06–4.13 (m, 4H,  $\text{OCH}_2$  of ethyl and N-O- $\text{CH}_2$ ), 4.54 (m, 1H, 3-CH), 4.66 (m, 1H, 16-CH), 6.56 (s, 1H, 6-CH).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz);  $\delta$  14.55 (C21), 14.98 ( $\text{CH}_3$ , ethyl), 17.04 (C18), 17.45 (C27), 18.28 (C19), 20.88 (C11), 21.65 (C3-acetate  $\text{CH}_3$ ), 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 ( $\text{CH}_2$ , ethyl), 60.76 (5' $\text{CH}_2$ ), 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 $\beta$ ,25R)-3 $\beta$ -acetoxy-spirost-5-en-7-iminoxy-ethylhexanoate (9)

**9:** Yield = 217 mg (84%), mp = 89–91 °C;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  0.72 (s, 3H, 18- $\text{CH}_3$ ), 0.92 (d, 3H, 27- $\text{CH}_3$ ,  $J = 6.9$  Hz), 1.11 (s, 3H, 19- $\text{CH}_3$ ), 1.19 (t, 3H,  $\text{CH}_3$ , ethyl ester,  $J = 7.1$  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,  $\text{CH}_3\text{COO}$ , 3-acetate), 3.34 (m, 2H, 26- $\text{CH}_2$ ), 4.04–4.09 (q, 4H,  $\text{OCH}_2$  of ethyl and N-O- $\text{CH}_2$ ,  $J = 7.1$  Hz), 4.46 (m, 1H, 3-CH), 4.60 (m, 1H, 16-CH), 6.52 (s, 1H, 6-CH).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz);  $\delta$  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  $\text{C}_{37}\text{H}_{57}\text{NO}_7$ : 628.3  $[\text{M}+\text{H}]^+$ .

## 2.2.9. (22 $\beta$ ,25R)-3 $\beta$ -acetoxy-spirost-5-en-7-iminoxy-ethylheptanoate (10)

**10:** Yield = 243 mg (92%), oil;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ),  $\delta$  0.78 (s, 3H, 18- $\text{CH}_3$ ), 0.96 (d, 3H, 27- $\text{CH}_3$ ,  $J = 7.0$  Hz), 1.11 (s, 3H, 19- $\text{CH}_3$ ), 1.23 (t, 3H,  $\text{CH}_3$ , ethyl,  $J = 7.5$  Hz), 1.23–2.39 (m, 35H, rest of the  $1 \times \text{CH}_3$ ,  $13 \times \text{CH}_2$  and  $6 \times \text{CH}$ ), 2.02 (s, 3H,  $\text{CH}_3\text{COO}$ , 3-acetate), 3.39 (m, 2H, 26- $\text{CH}_2$ ), 4.08–4.13 (m, 4H,  $\text{OCH}_2$  of ethyl and N-O- $\text{CH}_2$ ,  $J = 8.0$  Hz, 5.3 Hz), 4.46 (m, 1H, 3-CH), 4.60 (m, 1H, 16-CH), 6.45 (s, 1H, 6-CH);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 75 MHz);  $\delta$  14.26, 14.68, 16.63, 17.12, 17.95, 20.57, ESI Mass (MeOH) for  $\text{C}_{38}\text{H}_{59}\text{NO}_7$ : 664.7  $[\text{M}+\text{Na}]^+$ ; 681.3  $[\text{M}+\text{K}]^+$ ; ESI-HRMS for  $\text{C}_{38}\text{H}_{60}\text{NO}_7$  calc: 642.4370, observed: 642.4360; IR (KBr,  $\text{cm}^{-1}$ ): 2929, 1735, 1460, 1176.

## 2.2.10. Saponification of esters (5–10) to get acid derivatives (11–16)

**2.2.10.1. Synthesis of (22 $\beta$ ,25R)-3 $\beta$ -hydroxy-spirost-5-en-7-iminoxy-ethanoic acid (11).** Ester **6** (100 mg, 0.17 mmol) was taken in 1.2% aqueous methanolic alkali (water:MeOH = 1:9). The reaction mixture was stirred at room temperature for 2 h. Solvent was removed *in vacuo* and water was added, acidified with dil. HCl (5%, 10 mL), 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 = 87 mg (91%), mp = 145–146 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ 0.78 (s, 3H, 18-CH<sub>3</sub>), 0.95 (d, 3H, 27-CH<sub>3</sub>, *J* = 6.9 Hz), 1.14 (s, 3H, 19-CH<sub>3</sub>), 1.19–2.62 (m, 25H, rest of the 1×CH<sub>3</sub>, 8×CH<sub>2</sub> and 6×CH of steroidal ring), 3.42 (m, 2H, 26-CH<sub>2</sub>), 4.54 (s, 2H, N-OCH<sub>2</sub>), 4.56 (m, 2H, 3-CH & 16-CH), 6.54 (s, 1H, 6-CH). ESI Mass (MeOH) for C<sub>29</sub>H<sub>43</sub>NO<sub>6</sub>: 502.4 [M+H]<sup>+</sup>, 524.3 [M+Na]<sup>+</sup>, 540.4 [M+K]<sup>+</sup>; IR (KBr, cm<sup>-1</sup>): 2947, 1638, 1451, 1241.

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

**12:** Yield = 82 (86%), mp = 114–116 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ 0.72 (s, 3H, 18-CH<sub>3</sub>), 0.92 (d, 3H, 27-CH<sub>3</sub>, *J* = 6.6 Hz), 1.22 (s, 3H, 19-CH<sub>3</sub>), 1.27–2.66 (m, 27H, rest of the 1 × CH<sub>3</sub>, 9 × CH<sub>2</sub> and 6 × CH of steroidal ring), 2.11 (s, 3H, CH<sub>3</sub>COO, Acetate), 3.38 (m, 2H, 26-CH<sub>2</sub>), 3.79 (bs, 2H, N-O-CH<sub>2</sub>), 4.13 (m, 1H, 3-CH), 4.32 (m, 1H, 16-CH), 6.46 (s, 1 H, 6-CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz); δ 14.63 (C21), 16.91 (C18), 17.46 (C27), 18.28 (C19), 20.92 (C11), 21.96 (C3-acetate CH<sub>3</sub>), 29.15 (C24), 29.58 (C2), 30.62 (2'-CH<sub>2</sub>), 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<sub>2</sub>), 62.31 (C17), 67.09 (C26), (2'-CH<sub>2</sub>), 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<sub>32</sub>H<sub>47</sub>NO<sub>7</sub>: 580.2 [M-Na]<sup>+</sup>.

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

**13:** Yield = 73 (83%), mp = 147–149 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ 0.68 (s, 3H, 18-CH<sub>3</sub>), 0.90 (d, 3H, 27-CH<sub>3</sub>, *J* = 6.9 Hz), 1.11 (s, 3H, 19-CH<sub>3</sub>), 1.15–2.57 (m, 29H, rest of the 1 × CH<sub>3</sub>, 10 × CH<sub>2</sub> and 6 × CH of steroidal ring), 3.42 (t, 2H, 26-CH<sub>2</sub>, *J* = 6.0 Hz), 4.05 (t, 2H, N-OCH<sub>2</sub>, *J* = 6.3 Hz), 4.56 (m, 2H, 3-CH & 16-CH), 6.54 (s, 1H, 6-CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz); δ 14.65 (C21), 15.05 (CH<sub>3</sub>, 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<sub>2</sub>, ethyl), (C26), 71.01 (2'-CH<sub>2</sub>), 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 C<sub>31</sub>H<sub>47</sub>NO<sub>6</sub>: 529.4 [M]<sup>+</sup>.

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

**14:** Yield = 79 mg (89%), mp = 159–162 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ 0.68 (s, 3H, 18-CH<sub>3</sub>), 0.90 (d, 3H, 27-CH<sub>3</sub>, *J* = 5.4 Hz), 1.11 (s, 3H, 19-CH<sub>3</sub>), 1.15–2.57 (m, 31H, rest of the 1 × CH<sub>3</sub>, 11 × CH<sub>2</sub> and 6 × CH of steroidal ring), 3.42 (t, 2H, 26-CH<sub>2</sub>, *J* = 6.3 Hz), 4.54 (s, 2H, N-OCH<sub>2</sub>), 4.56 (m, 2H, 3-CH & 16-CH), 6.54 (s, 1H, 6-CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz); δ 14.65 (C21), 15.05 (CH<sub>3</sub>, 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<sub>2</sub>, ethyl), (C26), 71.01 (2'-CH<sub>2</sub>), 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 C<sub>34</sub>H<sub>51</sub>NO<sub>7</sub>: 544.3 [M+H]<sup>+</sup>; IR (KBr, cm<sup>-1</sup>): 2947, 1737, 1638, 1451, 1241.

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

**15:** Yield = 87 mg (91%), mp = 155–157 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ 0.72 (s, 3H, 18-CH<sub>3</sub>), 0.93 (d, 3H, 27-CH<sub>3</sub>, *J* = 6.6 Hz), 1.13 (s, 3H, 19-CH<sub>3</sub>), 1.18–2.78 (m, 24H, rest of the 1 × CH<sub>3</sub>, 8 × CH<sub>2</sub> and 6 × CH of steroidal ring), 3.42 (m, 2H, 26-CH<sub>2</sub>), 3.44 (t, 2H, N-OCH<sub>2</sub>, *J* = 6.3 Hz), 4.33 (m, 2H, 3-CH & 16-CH), 6.41 (s, 1H, 6-CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 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 C<sub>33</sub>H<sub>51</sub>NO<sub>6</sub>: 558.3 [M+H]<sup>+</sup>.

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

**16:** Yield = 80 mg (84%), mp = 237–239 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ 0.77 (s, 3H, 18-CH<sub>3</sub>), 0.97 (d, 3H, 27-CH<sub>3</sub>, *J* = 7.0 Hz), 1.14 (s, 3H, 19-CH<sub>3</sub>), 1.24–2.45 (m, 35H, rest of the 1 × CH<sub>3</sub>, 13 × CH<sub>2</sub> and 6 × CH of steroidal ring), 3.46 (bs, 2H, 26-CH<sub>2</sub>), 4.02 (t, 2H, N-OCH<sub>2</sub>, *J* = 6.5 Hz), 4.46 (m, 2H, 3-CH & 16-CH), 6.43 (s, 1H, 6-CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz); 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 C<sub>34</sub>H<sub>53</sub>NO<sub>6</sub>: 594.7 [M+Na]<sup>+</sup>, 610.5 [M+K]<sup>+</sup>; Negative mode: 570.9 [M-H]<sup>-</sup>. ESI-HRMS calc for C<sub>34</sub>H<sub>54</sub>NO<sub>6</sub>: 572.3914, found, 572.3912; IR (KBr, cm<sup>-1</sup>): 2947, 1737, 1638, 1451, 1241.

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

Benzyltriphenylphosphonium bromide (Wittig salt, 1 g, 2.1 mmol) was stirred in dry toluene (15 mL). To this sodium hydride (100 mg, 4 mmol) was added and further stirred for 10 min. 7-Keto derivative **3** (250 mg, 0.53 mmol) was added to it and reaction mixture was refluxed for 2 h. On completion water (20 mL) 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 = 78 mg (89%), mp = 182–185 °C; <sup>1</sup>H NMR (CDCl<sub>3</sub>), δ 0.76 (s, 3H, 18-CH<sub>3</sub>), 0.95 (d, 3H, 27-CH<sub>3</sub>, *J* = 6.9 Hz), 1.14 (s, 3H, 19-CH<sub>3</sub>), 1.19–2.29 (m, 19H, rest of the 1×CH<sub>3</sub>, 5×CH<sub>2</sub> and 6×CH of steroidal ring), 2.52 (t, 1H, 2-CH<sub>2</sub>, *J* = 6.3 Hz), 2.87 (t, 1H, 2-CH<sub>2</sub>), 3.38 (m, 2H, 26-CH<sub>2</sub>), 4.41 (q, 1H, 16-CH, *J* = 4.6 Hz), 5.58 (s, 1H, 6-CH), 6.14 (m, 2H, 3-CH and 4-CH). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 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 C<sub>27</sub>H<sub>38</sub>O<sub>3</sub>: 411.2 [M+H]<sup>+</sup>; ESI-HRMS for C<sub>27</sub>H<sub>39</sub>O<sub>3</sub> [M+H]<sup>+</sup>, 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 × 100 mm, 1.7 μm) C<sub>18</sub> 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: CH<sub>3</sub>CN- 70:30; *mobile phase-II*: Water: CH<sub>3</sub>OH- 80:20; run time-12 min; flow rate-0.3 ml/min). The injection volume was 2 μL. The column elution was monitored in the range of 190–400 nm using photodiode array detector (A16UPD0038, ACQUITY Waters, Cell volume-500 nL). 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 CO<sub>2</sub> incubator (New Brunswick/Eppendorff, Germany) under 5% CO<sub>2</sub> 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<sup>4</sup> cells/well were added and incubated overnight at 37 °C in 5% CO<sub>2</sub>. Next day (at 80% confluency), serial dilutions of test compound were added to the wells. Untreated cells served as control. After 48 h, cells were fixed with ice-cold 50% (w/v) trichloroacetic acid (100 µL/well) for 1 h at 4 °C. Cells were then stained with SRB dye (0.4% w/v in 1% acetic acid, 50 µL/well), washed and air-dried. Bound dye was solubilised with 10 mM Tris base (150 µL/well) and absorbance was read at 540 nm on a plate reader (Biotek, USA). The cytotoxic effect was calculated as;

% inhibition in cell growth = [1 – (Absorbance of treated cells/Absorbance of untreated cells) × 100]. Determination of 50% inhibitory concentration (IC<sub>50</sub>) 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<sup>5</sup> cells per well were seeded and grown overnight at 37 °C in 5% CO<sub>2</sub>. After treating with compound **16** for different time points, cells were harvested by trypsinization and fixed with ice-cold 70% ethanol for 30 min at 4 °C. The pellets were washed with PBS and re-suspended in a solution containing propidium iodide (20 mg/mL), TritonX100 (0.1%) and RNase (1 mg/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 (30 min, in ice) with cold M-PER (mammalian protein extraction reagent) supplemented with protease inhibitor cocktail. Equal amount of proteins (25 µg) 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; 20 mM Tris-HCl, pH 7.6, 137 mM NaCl) containing 0.1% Tween20 (TBS-T) for 1 h 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 1 h 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 1.0 mL 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<sup>6</sup> 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 CO<sub>2</sub> incubator at 37 °C with 5% CO<sub>2</sub> 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 1 µg/mL and 10 µg/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; 0.5 µg/mL). After incubation with LPS for 24 h, 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 (100 µL/well) with specific mouse TNF-α and IL-6 capture antibody respectively and incubated overnight at 4°C. The plate was blocked with 200 µL/well assay diluents. Culture supernatant and standard (100 µL) were added into the appropriate coated wells and incubated for 2 h at room temperature (20–25 °C). After incubation, the plates were washed thoroughly 5 times with wash buffer. 100 µL of detecting solution (detection antibody and streptavidin HRP) were added into each well. Plates were sealed and incubated for 1 h at RT and then the plates were washed thoroughly 5 times with wash buffer. 100 µL of tetramethylbenzidine (TMB) substrate solution were added to each well and incubated (without plate sealer) for 30 min at room temperature in the dark. 50 µL of stop solution (2 N H<sub>2</sub>SO<sub>4</sub>) 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:

% inhibition = 100 × (concentration of vehicle control-concentration of test treatment)/OD of concentration of vehicle control, 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 12 h. 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 1000 mg/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 analyzed 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.

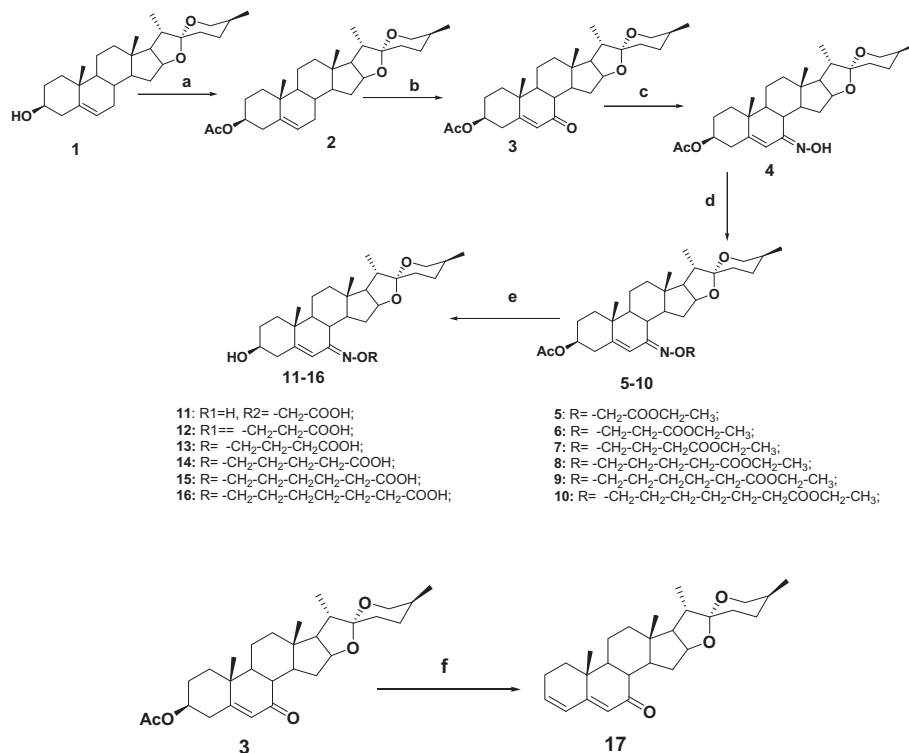
### 3. Results

#### 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-CrO<sub>3</sub> 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 CrO<sub>3</sub> 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 (Ph<sub>3</sub>P-CH<sub>2</sub>-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].

Compound **17** was obtained as white amorphous powder. Its structure was established by spectroscopy. Its molecular formulae C<sub>27</sub>H<sub>38</sub>O<sub>3</sub> was determined by its <sup>13</sup>C NMR and ESI-HR-MS at *m/z* 411.2872 [M+H]<sup>+</sup> (Calcd. 411.2899). In <sup>1</sup>H NMR acetyl protons were absent which was further confirmed in <sup>13</sup>C NMR ( $\delta$  21.6 and 170.6 both absent). <sup>13</sup>C NMR showed total 27 distinct carbons (four CH<sub>3</sub>, eight CH<sub>2</sub>, 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  $\delta$  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



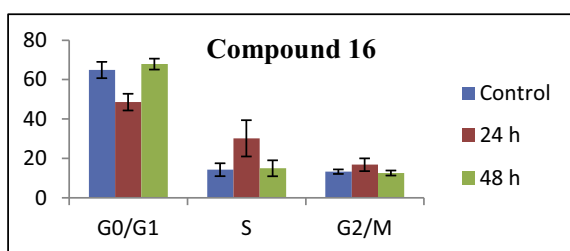
**Scheme 1.** Reagents and conditions: (a) Ac<sub>2</sub>O, dry pyridine, dry CHCl<sub>3</sub>, RT, 2 h, 91%; (b) Dry DCM, dry Pyridine, CrO<sub>3</sub>, 4 h, 68%; (c) dry pyridine, NH<sub>2</sub>OH-HCl, reflux, 2 h, 96%; (d) dry THF, NaH, ethylbromo ester, RT, 0.5 h–2 h, 70–92%; (e) 1.2% KOH in water:MeOH (1:9), RT, 1–2 h, 84–91%; (f) dry toluene, PPh<sub>3</sub>, CH<sub>2</sub>-Ar, NaH, reflux, 2–4 h, 84–91%.

**Table 1**

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

Compound name	IC <sub>50</sub> ( $\mu$ M) (Mean $\pm$ SE) <sup>a</sup>					
	FaDu	A549	DLD1	MCF-7	DU145	PC3
<b>9</b>	33.30 $\pm$ 5.89	>50	35.49 $\pm$ 2.66	35.10 $\pm$ 3.54	31.52 $\pm$ 2.56	–
<b>10</b>	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	–
<b>11</b>	31.64 $\pm$ 6.02	29.04 $\pm$ 3.45	31.22 $\pm$ 1.60	33.34 $\pm$ 0.98	33.03 $\pm$ 0.60	–
<b>13</b>	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	–
<b>14</b>	<b>16.56 <math>\pm</math> 1.57</b>	<b>17.05 <math>\pm</math> 3.61</b>	<b>16.01 <math>\pm</math> 1.88</b>	<b>15.59 <math>\pm</math> 1.89</b>	<b>14.67 <math>\pm</math> 1.26</b>	<b>21.85 <math>\pm</math> 0.53</b>
<b>16</b>	<b>15.21 <math>\pm</math> 0.90</b>	<b>15.77 <math>\pm</math> 2.22</b>	<b>14.55 <math>\pm</math> 0.64</b>	<b>15.77 <math>\pm</math> 1.98</b>	<b>12.82 <math>\pm</math> 1.26</b>	<b>25.39 <math>\pm</math> 1.70</b>
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).

<sup>a</sup> IC<sub>50</sub> > 50  $\mu$ M inactive.**Fig. 2.** Cell cycle analysis of analogue **16** in DU-145 cells.

and F. With all these observations the structure of transformed product **17** was confirmed as (22 $\beta$ ,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 5.811 min; *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.

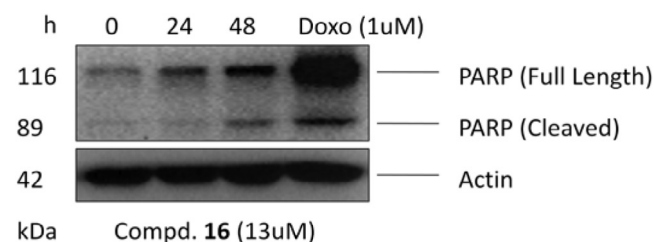
## 3.2. Pharmacology

### 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<sub>50</sub> < 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<sub>50</sub> = 12.8  $\mu$ M) against prostate cancer cell line DU-145.

### 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

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

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  $\mu$ M concentration. In 24 h incubation S phase arrest was quite significant but there was no such effect at 48 h incubation (Fig. 2).

### 3.2.3. Western blot experiment

In the present study, we found cleavage of PARP in DU-145 cells at 24 h exposure to the diosgenin analogue **16** at 13  $\mu$ M concentration implying activation of caspases by the molecule to trigger apoptosis (Fig. 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- $\alpha$  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- $\alpha$  (31.8–47.7%) at 1  $\mu$ g/mL and 10  $\mu$ 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.

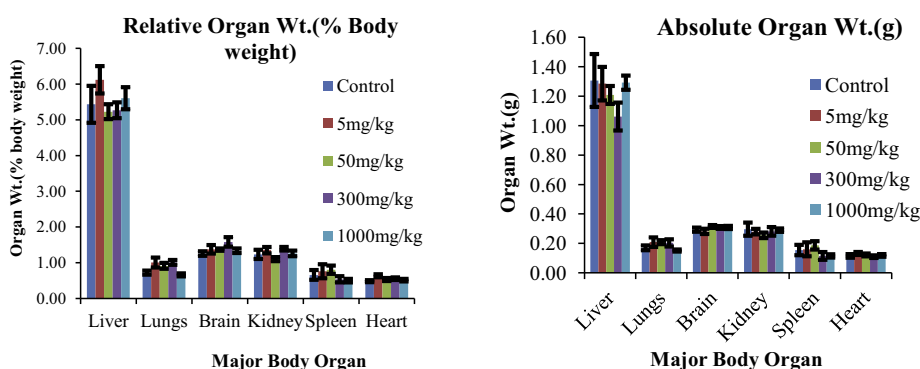
### 3.2.5. Safety studies

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 1000 mg/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 (Fig. 4) except serum albumin and SGOT levels. Serum albumin level was found to be significantly higher (Table 3) in the group of the animals treated with the test compound at 1000 mg/kg body weight compare to the control while SGOT level was found to be significantly higher in the group of animals treated with the test compound at 1000 mg/kg body weight compare to control and all other treatment groups. Animals

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

Compound	LPS (0.5 µg/mL)	Dose (µg/mL)	TNF-α (pg/mL)	TNF-α % inhibition	IL-6 (pg/mL)	IL-6 % inhibition
Normal	–	–	24.66 ± 0.72	NA	288.33 ± 85.11	NA
Vehicle	0.5 µg/mL	–	153 ± 6.17 <sup>#</sup>	00	2733 ± 29.40 <sup>#</sup>	00
<b>10</b>	0.5 µg/mL	1	104.33 ± 7.21 <sup>***</sup>	31.80 ± 4.71	2354 ± 61.69 <sup>**</sup>	13.86 ± 2.25
		10	94.16 ± 2.42 <sup>***</sup>	38.45 ± 1.58	2295 ± 55.01 <sup>***</sup>	16.02 ± 2.01
<b>13</b>	0.5 µg/mL	1	101.33 ± 2.20 <sup>***</sup>	33.76 ± 1.44	2353.66 ± 20.40 <sup>**</sup>	13.02 ± 0.74
		10	98.16 ± 4.47 <sup>***</sup>	35.83 ± 2.92	2261.66 ± 84.84 <sup>***</sup>	17.24 ± 3.10
<b>14</b>	0.5 µg/mL	1	100.66 ± 9.95 <sup>***</sup>	<b>34.20 ± 6.50</b>	2339 ± 40.46 <sup>**</sup>	14.41 ± 1.48
		10	80 ± 4.19 <sup>***</sup>	<b>47.71 ± 2.74</b>	2095.33 ± 88.64 <sup>***</sup>	<b>23.33 ± 3.24</b>
<b>16</b>	0.5 µg/mL	1	97 ± 7.21 <sup>***</sup>	<b>36.60 ± 4.71</b>	2251 ± 78.83 <sup>***</sup>	17.63 ± 2.88
		10	89.16 ± 11.98 <sup>***</sup>	<b>41.72 ± 7.83</b>	2193.66 ± 45.60 <sup>***</sup>	<b>19.73 ± 1.66</b>
Diosgenin	0.5 µg/mL	1	117.50 ± 5.77 <sup>*</sup>	23.20 ± 3.77	2397.66 ± 75.51 <sup>†</sup>	12.26 ± 2.76
		10	95.16 ± 5.08 <sup>***</sup>	37.79 ± 3.32	2048 ± 36.75 <sup>***</sup>	25.06 ± 1.34
Dexamethasone	0.5 µg/mL	1	59.5 ± 7.48 <sup>***</sup>	61.11 ± 4.89	925.66 ± 75.13 <sup>***</sup>	66.13 ± 2.74
		10	35.83 ± 9.53 <sup>***</sup>	76.57 ± 6.23	694 ± 86.49 <sup>***</sup>	74.60 ± 3.16

Normal vs Vehicle; <sup>#</sup>P < 0.05; Vehicle vs Treatment; <sup>ns</sup>-not significant, <sup>\*</sup>P < 0.05; n = 3.



**Fig. 4.** 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).

**Table 3**  
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).

Parameters	Control	5 mg/kg	50 mg/kg	300 mg/kg	1000 mg/kg
<i>Dose of compound 16 at mg/kg body weight as a single oral dose</i>					
Body wt. 7th 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 <sup>3</sup> )	6.29 ± 0.26	6.17 ± 0.29	6.28 ± 0.03	5.93 ± 0.32	5.54 ± 0.34
WBC (thousands/mm <sup>3</sup> )	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 <sup>a</sup>
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 <sup>*</sup>
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

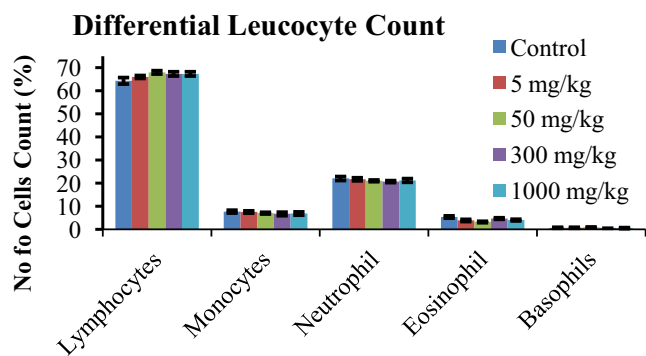
on gross pathological study did not show any change in any of the organs studied including their absolute and relative weight. Since, rise in serum albumin level indicates damage of liver function and burden on vital function of kidney and immune system and additional significant rise in the level of SGOT indicate that the test compound **16** found to be toxic at 1000 mg/kg body weight. Therefore, the experiment showed that compound **16** is well tolerated by the Swiss albino mice up to the dose level of 300 mg/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 1000 mg/kg (Fig. 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





**Fig. 5.** 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).

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 24 h 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 24 h 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- $\kappa$ B and many other inflammatory mediators like cytokines (TNF $\alpha$  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- $\alpha$  and IL-6 expressions moderately at  $1 \mu g/mL$  and  $10 \mu g/mL$  concentrations. This additional property is very important and desirable in anticancer agents. Diosgenin and its analogues have been reported to possess anti-inflammatory property previously [38].

In acute oral toxicity compound **16** was found to be safe up to 300 mg/kg oral dose. But at a higher dose of 1000 mg/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.

## 5. Conclusion

Present study has provided several novel analogues of diosgenin at C7 position. Two of the analogues possessed significant anticancer and anti-inflammatory 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.

## Acknowledgements

Authors are thankful to the Directors of CSIR-CDRI and CSIR-CIMAP for constant encouragement and support. Mrs. Namita Gupta is acknowledged for help in spectral data. The financial support from NMPB through GAP-333 is duly acknowledged. TWAS-CSIR fellowship to Mr. A. A. Hamid is duly acknowledged.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.steroids.2017.01.001>.

## References

- [1] J.R. Hanson, Steroids: partial synthesis in medicinal chemistry, *Nat. Prod. Rep.* 27 (2010) 887–899.
- [2] A. Gupta, B.S. Kumar, S. Arvind, A.S. Negi, Current status on development of steroids as anticancer agents, *J. Steroid Biochem. Mol. Biol.* 137 (2013) 242–270.
- [3] (a) K.G. Ramawat, S. Dass, M. Mathur, *Herbal Drugs, Ethnomedicine to Modern Medicine*, Springer Science & Business Media, 2008. ISBN: 3540791167, 9783540791164;  
(b) D. Lednicer, *Steroid Chemistry at a Glance*, John Wiley & Sons Ltd., West Sussex, UK, 2011, p. 23;  
(c) C. Yan, T. You-Mei, Y. Su-Lan, H. Yu-Wei, K. Jun-Ping, L. Bao-Lin, Y. Bo-Yang, Advances in the pharmacological activities and mechanism of diosgenin, *Chin. J. Nat. Med.* 13 (8) (2015) 0578–0587.
- [4] (a) S. Das, K.K. Dey, G. Dey, I. Pal, A. Majumder, S. Maiti Choudhury, S.C. Kundu, M. Mandal, Antineoplastic and apoptotic potential of traditional medicines thymoquinone and diosgenin in squamous cell carcinoma, *PLoS One* 7 (10) (2012) e46641;  
(b) P. Aumsuwan, S.I. Khan, I.A. Khan, Z. Ali, B. Avula, L.A. Walker, Z. Shariat-Madar, W.G. Helferich, B.S. Katzenellenbogen, A.K. Dasmahapatra, The anticancer potential of steroidal saponin, dioscin, isolated from wild yam (*Dioscorea villosa*) root extract in invasive human breast cancer cell line MDA-MB-231 in vitro, *Arch. Biochem. Biophys.* 591 (2015) 98–110.
- [5] Y. Chen, X. Xu, Y. Zhang, K. Liu, F. Huang, B. Liu, J. Kou, Diosgenin regulates adipokine expression in perivascular adipose tissue and ameliorates endothelial dysfunction via regulation of AMPK, *J. Steroid Biochem. Mol. Biol.* 155 (2016) 155–165.

- [6] G.H. Gong, Y. Qin, W. Huang, Anti-thrombosis effect of diosgenin extract from *Dioscorea zingiberensis* C.H. Wright *in vitro* and *in vivo*, *Phytomedicine* 18 (2011) 458–463.
- [7] (a) D.H. Jung, H.J. Park, H.E. Byun, Y.M. Park, T.W. Kim, B.O. Kim, S.H. Um, S. Pyo, Diosgenin inhibits macrophages-derived inflammatory mediators through downregulation of CK2, JNK, NF-kappaB and AP-1 activation, *Int. Immunopharmacol.* 10 (2010) 1047–1054;  
(b) C.H. Huang, D.Z. Liu, T.R. Jan, Diosgenin a plant derived saponin, enhances regulatory T-cell immunity in the intestine of mice with food allergy, *J. Nat. Prod.* 73 (2010) 1033–1037.
- [8] Y.J. Wang, K.L. Pan, T.C. Hsieh, T.Y. Chang, W.H. Lin, J.T.A. Hsu, Diosgenin, a plant derived saponin, exhibits antiviral activity *in vitro* against hepatitis C virus, *J. Nat. Prod.* 74 (2011) 580–604.
- [9] S. Ghosh, P. More, A. Derle, A.B. Patil, P. Markad, A. Asok, N. Kumbhar, M.L. Shaikh, B. Ramanamurthy, V.S. Shinde, D.D. Dhavale, B.A. Chopade, Diosgenin from *Dioscorea bulbifera*: novel hit for treatment of type II diabetes mellitus with inhibitory activity against  $\alpha$ -amylase and  $\alpha$ -glucosidase, *PLoS One* 9 (9) (2014) e106039.
- [10] X.J. Qin, D.J. Sun, C.X. Chen, Y. Hua, L. He, H.Y. Liu, Steroidal saponins with antimicrobial activity from stems and leaves of *Paris polyphylla* var. *Yunnanensis*, *Steroids* 77 (2012) 1242–1248.
- [11] C. Corbiere, B. Liagre, F. Terro, J.L. Beneytout, Induction of antiproliferative effect by diosgenin through activation of p53, release of apoptosis-inducing factor (AIF) and modulation of caspase-3 activity in different human cancer cells, *Cell Res.* 14 (2004) 188–196.
- [12] (a) P.S. Chen, Y.W. Shih, H.C. Huang, H.W. Cheng, Diosgenin, a steroidal saponin, inhibits migration and invasion of human prostate cancer PC-3 cells by reducing matrix metalloproteinase expression, *PLoS One* 6 (5) (2011) e20164;  
(b) F. Li, P.P. Fernandez, P. Rajendran, K.M. Hui, G. Sethi, Diosgenin, a steroidal saponin, inhibits STAT3 signaling pathway, leading to suppression of proliferation and chemosensitization of human hepatocellular carcinoma cells, *Cancer Lett.* 292 (2010) 197–207;  
(c) M.J. Kaskiw, M.L. Tassotto, J. Th'nga, Z.H. Jiang, Synthesis and cytotoxic activity of diosgenyl saponin analogues, *Bioorg. Med. Chem.* 16 (2008) 3209–3217;  
(d) M.J. Kaskiw, M.L. Tassotto, M. Mok, S.L. Tokar, R. Pycko, J. Th'nga, Z.H. Jiang, Structural analogues of diosgenyl saponins: synthesis and anticancer activity, *Bioorg. Med. Chem.* 17 (2009) 7670–7679;  
(e) S. Moalic, B. Liagre, C. Corbiere, A. Bianchi, M. Sauca, R. Bordji, J.L. Beneytout, A plant steroid, diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells, *FEBS Lett.* 506 (2001) 225–230.
- [13] Y.J. Kang, H.J. Chung, J.W. Nam, H.J. Park, E.K. Seo, Y.S. Kim, D. Lee, S.K. Lee, Cytotoxic and antineoplastic activity of Timosaponin A-III for human colon cancer cells, *J. Nat. Prod.* 74 (2011) 701–706.
- [14] Q.Y. Tong, Q.B. Zhao, W. Huang, X.H. Wu, Cytotoxicity and apoptosis inducing effect of steroidal saponins from *Dioscorea zingiberensis* Wright against cancer cells, *Steroids* 77 (2012) 1219–1227.
- [15] Nomenclature of Steroids, IUPAC and international union of biochemistry-joint commission on biochemical nomenclature, *Pure Appl. Chem.* 61 (1989) 1783–1822.
- [16] A.A. Hamid, M. Hasanain, A. Singh, Omprakash, P.G. Vasudev, J. Sarkar, D. Chanda, F. Khan, O.O. Aiyelaagbe, A.S. Negi, Synthesis of novel anticancer agents through opening of spiroketal ring of diosgenin, *Steroids* 87 (2014) 108–118.
- [17] V. Pathak, I. Ahmad, A.K. Kahlon, M. Hasanain, S. Sharma, K.K. Srivastava, J. Sarkar, K. Shankar, A. Sharma, A. Gupta, Syntheses of 2-methoxyestradiol and eugenol template based diarylpropenes as non-steroidal anticancer agents, *RSC Adv.* 4 (66) (2014) 35171–35185.
- [18] O.A. Adaramoye, J. Sarkar, N. Singh, et al., Antiproliferative action of *Xylopiya aethiopica* fruit extract on human cervical cancer cells, *Phytother. Res.* 25 (2011) 1558–1563.
- [19] J. Sarkar, N. Singh, S. Meena, S. Sinha, Staurosporine induces apoptosis in human papillomavirus positive oral cancer cells at G2/M phase by disrupting mitochondrial membrane potential and modulation of cell cytoskeleton, *Oral Oncol.* 45 (2009) 974–979.
- [20] (a) S. Sharma, S.K. Chattopadhyay, M. Singh, D.U. Bawankule, S. Kumar, Novel chemical constituents with anti-inflammatory activity from the leaves of *Sesbania aculeata*, *Phytochemistry* 2014 (100) (2014) 132–140;  
(b) D.U. Bawankule, S.K. Chattopadhyay, A. Pal, K. Saxena, S. Yadav, U. Faridi, M.P. Darokar, A.K. Gupta, S.P.S. Khanuja, Modulation of inflammatory mediators by coumarinolignoids from *Cleome viscosa* in female Swiss albino mice, *Inflammopharmacology* 16 (2008) 272–277.
- [21] J.J. Allan, A. Damodaran, N.S. Deshmukh, K.S. Goudar, A. Amit, Safety evaluation of a standardized phytochemical composition extracted from *Bacopa monnieri* in Sprague-Dawley rats, *Food Chem. Toxicol.* 45 (2007) 1928–1937.
- [22] D. Chanda, K. Shanker, A. Pal, S. Luqman, D.U. Bawankule, D.N. Mani, M.P. Darokar, Safety evaluation of Trikatu, a generic Ayurvedic medicine in Charles Foster rat, *J. Toxicol. Sci.* 34 (1) (2008) 99–108.
- [23] (a) J. Raju, R. Mehta, Cancer chemopreventive and therapeutic effects of diosgenin, a food saponin, *Nutr. Cancer* 61 (2009) 27–35;  
(b) Z. Wang, Y. Cheng, N. Wang, D.M. Wang, Y.W. Li, F. Han, J.G. Shen, P. Yang de, X.Y. Guan, J.P. Chen, Dioscin induces cancer cell apoptosis through elevated oxidative stress mediated by downregulation of peroxiredoxins, *Cancer Biol. Ther.* 13 (2012) 138–147.
- [24] C.T. Chiang, T.D. Way, S.J. Tsai, J.K. Lin, Diosgenin a naturally occurring steroid suppresses fatty acid synthase expression in HER2-overexpressing breast cancer cells through modulating Akt, mTOR and JNK phosphorylation, *FEBS Lett.* 581 (2007) 5735–5742.
- [25] Y. Wang, Q.Y. He, J.F. Chiu, Dioscin induced activation of p38 MAPK and JNK via mitochondrial pathway in HL-60 cell line, *Eur. J. Pharmacol.* 735 (2014) 52–58.
- [26] World Cancer Report, International Agency for Research on Cancer, World Health Organization, 2014.
- [27] A.W. Hsing, A.P. Chokkalingam, Prostate cancer epidemiology, *Front. Biosci.* 11 (2006) 1388–1413.
- [28] (a) H. Hong, Y. Zhang, J. Sun, W. Cai, Positron emission tomography imaging of prostate cancer, *Amino Acids* 39 (1) (2009) 11–27;  
(b) M. Peyromaure, A. Valéri, X. Rebillard, P. Beuzeboc, P. Richaud, M. Soulié, L. Salomon, Characteristics of prostate cancer in men less than 50-year-old, *Prog. Urol.* 19 (11) (2009) 803–809.
- [29] B. Seruga, A. Ocana, I.F. Tannock, Drug resistance in metastatic castration-resistant prostate cancer, *Nat. Rev. Clin. Oncol.* 8 (1) (2011) 12–23.
- [30] A.G. Porter, R.U. Jänicke, Emerging roles of caspase-3 in apoptosis, *Cell Death Differ* 6 (1999) 99–104.
- [31] S.J. Riedl, Y. Shi, Molecular mechanisms of caspase regulation during apoptosis, *Nat. Rev. Mol. Cell Biol.* 5 (11) (2004) 897–907.
- [32] (a) M.S. Satoh, T. Lindahl, Role of poly(ADP-ribose) formation in DNA repair, *Nature* 356 (1992) 356–358;  
(b) M. Tewari, L.T. Quan, K. O'Rourke, S. Desnoyers, Z. Zheng, D.R. Beidler, G.G. Poiries, G. Salvesen, V.M. Dixit, Yama/ CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase, *Cell* 81 (1995) 801–809;  
(c) M. Germain, E.B. Affar, D. D'Amours, V.M. Dixit, G.S. Salvesen, G.G. Poirier, Cleavage of automodified poly(ADP-ribose) polymerase during apoptosis. Evidence for involvement of caspase-7, *J. Biol. Chem.* 274 (1999) 28379–28384.
- [33] D.S. Kim, B.K. Jeon, Y.E. Lee, W.H. Woo, Y.J. Mun, Diosgenin induces apoptosis in HepG cells through generation of reactive oxygen species and mitochondrial pathway, *Evidence Based Complement Alter Med.* (2012). 981675.
- [34] K. Collins, T. Jacks, N.P. Payleitch, The cell cycle and cancer, *Proc. Natl. Acad. Sci.* 94 (1997) 2776–2778.
- [35] L.H. Hartwell, T.A. Weinert, Checkpoint controls that ensures the order of the cell cycle events, *Science* 246 (1989) 629–634.
- [36] (a) F. Balkwill, A. Mantovani, Inflammation and cancer: back to Virchow?, *Lancet* 357 (2002) 539–545;  
(b) L.M. Coussens, Z. Werb, Inflammation and cancer, *Nature* 420 (2002) 860–867.
- [37] F.R. Greten, L. Eckmann, T.F. Greten, J.M. Park, L.J. Egan, M.F. Kagnoff, M. Karin, IKKbeta links inflammation and tumorigenesis in a mouse model of colitis associated cancer, *Cell* 118 (2004) 285–296.
- [38] M. Singh, A.A. Hamid, A.K. Maurya, O. Prakash, F. Khan, A. Kumar, O.O. Aiyelaagbe, A.S. Negi, D.U. Bawankule, Synthesis of diosgenin analogues as potential anti-inflammatory agents, *J. Steroid Biochem. Mol. Biol.* 143C (2014) 323–333.