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Synthesis of diosgenin analogues as potential anti-inflammatory agents

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ABSTRACT

We herein report the synthesis of diosgenin analogues from commercially available diosgenin as the starting material. The structures of newly synthesised compounds were confirmed by ^{1}H NMR, ^{13}C NMR and mass spectrometry. All analogues were evaluated for *in-vitro* cytotoxicity followed by anti-inflammatory profile against LPS-induced inflammation in primary peritoneal macrophages isolated from mice by quantification of pro-inflammatory (TNF-α, IL-6 and IL-1β) cytokines in cell culture supernatant using the ELISA technique. Among the synthesised analogues, analogue **15** [(E) 26-(3′,4′,5′-trimethoxybenzylidene)-furost-5en-3β-acetate)] showed significant anti-inflammatory activity by inhibiting LPS-induced pro-inflammatory cytokines in a dose-dependent manner without any cytotoxicity. Efficacy and safety of analogue **15** were further validated in an *in-vivo* system using LPS-induced sepsis model and acute oral toxicity in mice. Oral administration of analogue **15** inhibited the pro-inflammatory cytokines in serum, attenuated the liver and lung injury and reduced the mortality rate in sepsis mice. Acute oral toxicity study showed that analogue **15** is non-toxic at higher dose in BALB/c mice. Molecular docking study revealed the strong binding affinity of diosgenin analogues to the active site of the pro-inflammatory proteins. These findings suggested that analogue **15** may be a useful therapeutic candidate for the treatment of inflammatory diseases.

1. Introduction

Inflammation is a complex and an important host defence mechanism in response to different stimuli, such as pathogens, physical injury and chemical injury. At a damaged site, inflammation is initiated by migration of immune cells from blood vessels and release of mediators, followed by recruitment of inflammatory cells and release of reactive oxygen species, reactive nitrogen species and pro-inflammatory cytokines to eliminate foreign pathogens, resolving infection and repairing injured tissues [1]. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged release of inflammatory mediators lead to chronic inflammation. Inflammation is linked to a wide range of progressive diseases, including sepsis, auto-immune disorders, neurological diseases, metabolic disorder and cardiovascular disease [2,3] which impose severe social and financial burdens including poor quality of life, high health-care costs and substantial loss of productivity. Sepsis, a life-threatening disease with a high mortality rate, is accompanied by systemic inflammation with excessive production of pro-inflammatory cytokines including tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [4]. Lipopolysaccharide (LPS), an endotoxin and the outer membrane component of Gram-negative bacteria, is a major pathogenic factor in sepsis [5]. LPS has been established for inflammatory research because LPS induces systemic inflammation mimicking the initial clinical features of sepsis [6].

Steroids are very important class of anti-inflammatory agents (SAIA). They suppress immune response through inhibition of NF-κB and by suppression of pro-inflammatory cytokines. They also inhibit production of prostaglandins and leukotrienes. Some of the notable steroidal anti-inflammatory drugs are dexamethasone,

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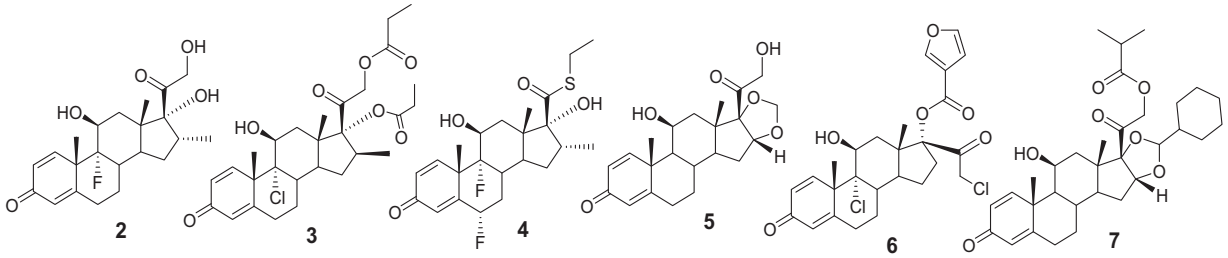

Fig. 1. Structures of standard anti-inflammatory steroid drugs; dexamethasone (**2**), beclometasone dipropionate (**3**), fluticasone (**4**), budesonide (**5**), mometasone (**6**) and ciclesonide (**7**).

beclometasone dipropionate, fluticasone, budesonide, mometasone, ciclesonide, etc. (Fig. 1). These are used for the management of various human disease conditions linked with inflammation [7].

The majority of natural molecules and their analogues showed potent anti-inflammatory activities and some established themselves as clinical agents for chronic inflammatory disease conditions. Therefore, plant-based natural compounds play a significant role in the development of anti-inflammatory drugs in the pharmaceutical industry which can serve as a good source of lead molecules suitable for further modification during the drug development process.

Diosgenin is a C27 spiroketal steroidal sapogenin abundantly available in nature. It is mainly present in the form of saponin in the plants including *Trigonella*, *Dioscorea* [8], *Costus* [9] and *Smilax* species [10]. In traditional medicine, it is used as an anti-hypercholesterolemia, antihypertriacylglycerolemia, antidiabetes and antihyperglycemia agent [11]. Several pharmacological reports reveal that diosgenin improves vascular function by increasing aortic eNOS expression in chronic renal failure model in rat [12], inhibits proliferation and induces apoptosis in a wide variety of cancer cell lines [13,14]. The antiproliferative and apoptotic properties are due to its ability to arrest the cell cycle, activate p53, release apoptosis-inducing factor, modulate caspase-3 activity [15] and due to inhibition of the ERK, JNK and phosphoinositide 3-kinase signalling pathways and nuclear factor kappa B activity (NF-κB) [16]. The NF-κB family of transcription factors has been increasingly recognised as a crucial player in many steps of cancer and inflammation [17]. Recently, it has been reported that diosgenin exhibits anti-inflammatory activity due to down-regulation of ICAM-1 expression through NFκB pathway [18]. In the present study, we have synthesised several analogues of diosgenin by modifying at spiroketal ring (Fig. 2) to examine its influences on inflammatory response; we validated the hypothesis using *in-vitro* and *in-vivo* bioassay techniques and it was further confirmed using *in-silico* study.

2. Materials and methods

2.1. General procedures

The starting substrate diosgenin (purity ~93%) was procured from Sigma Chemicals, USA. All the dry solvents were prepared as per standard methods. Reagents were used as such without any further purification. Reactions were monitored in Merck aluminium sheet silica gel thin layer plates (TLC, $60F_{254}$), visualised in UV-cabinet (λ_{max} = 254 and 365 nm) and further charred with 2% ceric sulphate in 10% aqueous sulphuric acid with subsequent heating at 80–100 °C. Melting points were determined in open capillaries in E-Z Melt melting point apparatus, Stanford Research System, USA. Solvents were evaporated under reduced pressure at 50 °C in Buchi Rotavapor. Compounds were purified through column chromatography over silica gel (Avra Chemicals, India, 100–200 mesh). NMR experiments were performed on Bruker Avance DRX 300 MHz spectrometer using tetramethylsilane (TMS, δ scale, 0.00 ppm) as internal standard. Splitting of peaks are abbreviated as s for singlet, d for doublet, t for triplet, q for quartet, bs for broad singlet and m for multiplet. ^{1}H and ^{13}C spectra are reported. Electrospray mass analysis was done on API 3000 Triple Quad LC–MS–MS (Applied Biosystem, USA) mass spectrometer after dissolving samples in methanol or acetonitrile. Nomenclature of diosgenin analogues has been done as per recommendations published by the Joint Commission on the Biochemical Nomenclature (JCBN) of IUPAC [19].

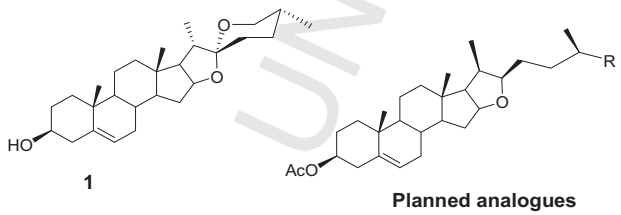

Fig. 2. Structures of diosgenin (1) and planned analogues.

2.2. Chemical synthesis

2.2.1. Synthesis of analogues ***8*** and ***17***

Acetylation was done as per reported method [20]. Substrate was taken in dry chloroform and pyridine at room temperature and acetic anhydride was added to it. Usual work-up was done after pouring into water. Q4

Analogue **8** (*3β-Hydroxy-(25R)-Spirost-5-en-3β-acetate*): Yield = 91%, mp = 193–196 °C; ^{1}H NMR ($CDCl_3$), δ 0.77 (s, 3H, 18-CH_3), 0.96 (d, 3H, 27-CH_3), 1.02 (s, 3H, 19-CH_3), 1.11–2.31 (m, 25H, rest of the 1×CH_3, 8×CH_2 and 6×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); δ 14.89, 16.64, 17.51, 19.69, 21.20, 21.74, 28.12, 29.19, 30.66, 31.78, 31.80, 32.21, 32.41, 37.10, 37.34, 38.47, 40.10, 40.63, 42.00, 42.68, 50.35, 56.82, 62.52, 67.19, 74.26, 81.16, 109.60, 122.72, 140.05, 170.82; ESI mass (MeOH): 457.3 $[M+H]^+$, 479.3 $[M+Na]^+$, 495.4 $[M+K]^+$. Q5

Analogue **17** (*Furost-5-en-3β, 26N-diacetoxy-26-aldoxime*)**:** Yield = 92%, mp = 90–93 °C, ^{1}H NMR ($CDCl_3$): δ 0.79 (s, 3H, 18-CH_3), 0.99 (s, 3H, 19-CH_3), 1.02–1.97 (m, 29H, rest of the 2×CH_3, 8×CH_2 and 7×CH of steroidal ring), 2.02 (s, 6H, 2×CH_3COO, 3 and 26-oxime acetates), 2.30 (bd, 2H, 7-CH_2, J = 6.9 Hz), 3.29 (bs, 1H, 22-CH), 4.29 (bs, 1H, 3-CH), 4.60 (bd, 1H, 16-CH), 5.36 (s, 1H, 6-CH), 7.50-7.53 (d, 1H, 26-CH, J = 8.1 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.81, 19.20, 19.72, 19.91, 21.03, 21.81, 26.33, 28.14, 30.08, 31.52, 31.96, 32.38, 32.59, 37.11, 37.39, 38.06, 38.28, 38.49, 39.77, 41.09, 50.40, 57.29, 65.32, 74.29, 83.83, 90.14, 122.74, 140.12, 163.46, 169.16, 170.95; ESI mass (MeOH): 551 $[M+K-1]^+$.

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*2.2.2. Synthesis of (25R) furost-5-en-, 3β-acetoxy, 26-ol (**9**)*

Diosgenin 3-acetate (200 mg, 0.44 mmol) was stirred in a 5 mL acetic acid. To this solution, sodium cyanoborohydride (200 mg, 3.17 mmol) was added in portions over a period of 30 min. After 2 h, when the reaction was complete, the reaction mixture was poured in ice-cool water, extracted with ethyl acetate (3 × 30 mL), washed with water and dried over anhydrous sodium sulphate. The organic layer was dried *in-vacuo* to get a crude mass, which was purified through column chromatography over silica gel using hexane-ethyl acetate as eluants. The desired alcohol **9** was obtained at 10–12% ethyl acetate–hexane as white crystalline solid.

Analogue **9:** Yield = 81%, mp = 121–122 °C; ^{1}H NMR ($CDCl_3$): δ 0.83 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.03–1.90 (m, 28H, rest of the 2×CH_3, 8×CH_2 and 6×CH of steroidal ring), 2.05 (s, 3H, CH_3COO, acetate), 2.35 (bd, 2H, 7-CH_2), 3.36 (bs, 1H, 22-CH), 3.48 (m, 2H, 27-CH_2OH), 4.34 (bs, 1H, 3-CH), 4.63 (bs, 1H, 16-CH), 5.40 (s, 1H, 6-CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.80, 17.00, 19.30, 19.69, 21.03, 21.77, 28.13, 30.46, 30.82, 31.94, 32.36, 32.59, 36.08, 37.07, 37.37, 38.28, 38.46, 39.78, 41.07, 50.39, 57.28, 65.48, 68.27, 74.28, 83.57, 90.71, 122.74, 140.04, 170.91; ESI mass (MeOH): 459.4 $[M+H]^+$, 481.3 $[M+Na]^+$, 497.4 $[M+K]^+$.

*2.2.3. Synthesis of furost-5-en-3β-acetoxy 26-al (**10**)*

Alcohol **9** (200 mg, 0.43 mmol) was taken in 10 ml methylene chloride. To this stirred solution, pyridinium chlorochromate (PCC, 200 mg, 0.93 mmol) was added and further stirred for an hour. On completion, solvent was evaporated and residue was dissolved in ethyl acetate. It was acidified with dil. HCl and washed with water. The organic layer was dried over anhydrous sodium sulphate and dried *in-vacuo*. The residue thus obtained was recrystallised with chloroform–hexane (1:3) to get aldehyde **10** as brown coloured solid.

Analogue **10:** Yield = 91%, mp = 119–123 °C; ^{1}H NMR ($CDCl_3$): δ 0.80 (s, 3H, 18-CH_3), 0.93 (s, 3H, 19-CH_3), 1.16–1.97 (m, 28H, rest of the 2×CH_3, 8×CH_2 and 6×CH of steroidal ring), 2.16 (s, 3H, CH_3COO, Acetate), 2.46 (bd, 2H, 7-CH_2), 3.45 (bs, 1H, 22-CH), 4.44 (bs, 1H, 3-CH), 4.73 (bd, 1H, 16-CH), 5.50 (s, 1H, 6-CH), 9.75 (s, 1H, 26-CHO). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 13.78, 16.79, 19.22, 19.71, 21.03, 21.77, 28.15, 30.07, 31.11, 31.96, 32.37, 32.59, 37.09, 37.39, 38.26, 38.48, 39.77, 41.08, 46.72, 50.41, 57.29, 65.44, 74.28, 83.28, 90.11, 122.73, 140.09, 170.91, 205.54; ESI mass (MeOH): 457.3 $[M+H]^+$, 479.3 $[M+Na]^+$, 495.4 $[M+K]^+$; ESI-HRMS: 457.33181 (calculated), and 457.3311 (observed) for $C_{29}H_{45}O_4$.

*2.2.4. Synthesis of 27-nor- furost-5-en- 3β-acetoxy-25-one (**11**)*

Aldehyde **10** (200 mg, 0.44 mmol) was taken in 10 mL ethanol. To this stirred solution, 3,4,5-trimethoxyaniline (200 mg, 1.09 mmol) was added and further stirred for 2 h at room temperature. The solvent was evaporated and the residue was dissolved in ethyl acetate and washed with water. The organic phase was dried over anhydrous sodium sulphate and dried *in-vacuo* to get a residue. It was purified through silica gel column eluting with ethyl acetate:hexane. The desired ketone **11** was obtained at 8–10% ethyl acetate–hexane as creamish white solid.

Analogue **11:** Yield = 84%, mp = 138–140 °C; ^{1}H NMR ($CDCl_3$): δ 0.79 (s, 3H, 18-CH_3), 0.98 (s, 3H, 19-CH_3), 1.02–1.87 (m, 23H, rest of the 1×CH_3, 8×CH_2 and 4×CH of steroidal ring), 1.95 (s, 3H, CH_3COO, acetate), 2.13 (s, 3H, 26-CH_3CO), 2.32 (d, 1H, 7-CH_2, J = 6.3 Hz) 2.51–2.63 (bd, 2H, 24-CH_2), 3.26–3.29 (bs, 1H, 22-CH), 4.24–4.29 (bs, 1H, 16-CH), 4.57 (bd, 1H, 3-CH), 5.35 (s, 1H, 6-CH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.80, 19.01, 19.71, 21.02, 21.80, 27.44, 28.13, 30.33, 31.95, 32.37, 32.55, 37.10, 37.38, 38.24, 38.48, 39.75, 41.09, 41.28, 50.38, 57.27, 65.39, 74.28, 83.69, 89.53, 122.72, 140.12, 170.95, 209.24; ESI mass (MeOH): 443.3 $[M+H]^+$, 465.4 $[M+Na]^+$, 481.3 $[M+K]^+$; ESI-HRMS: 443.31616 (calculated) and 443.3139 (observed) for $C_{28}H_{43}O_4$.

*2.2.5. Synthesis of Wittig products **12–15***

*2.2.5.1. Synthesis of (22β)-(E)-26-benzylidene-3β-yl-furost-5-en-3-acetate (**12**).* Sodium hydride (200 mg, 8.3 mmol) was washed with dry hexane and taken in 10 mL dry toluene. To this Wittig salt (150 mg, 0.34 mmol) was added and refluxed for 20 min. To this aldehyde **10** (100 mg, 0.22 mmol) was added and further refluxed for 4 h. Toluene was evaporated under vacuum and residue was taken in ethyl acetate, washed with water and dried over anhydrous sodium sulphate. The organic layer was dried *in-vacuo* to get a crude mass, which was purified through silica gel column eluting with ethyl acetate–hexane. The desired product was obtained as yellowish viscous liquid.

Analogue **12:** Yield = 68%, oil; ^{1}H NMR ($CDCl_3$): δ 0.73 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 1.02–1.95 (m, 26H, rest of the 2×CH_3, 7×CH_2 and 6×CH of steroidal ring), 1.99 (s, 3H, CH_3COO, acetate), 2.23 (d, 2H, 4-CH_2 J = 5.4 Hz), 2.30 (bd, 2H, 7-CH_2), 3.23 (bd, 1H, 22-CH), 4.22 (bs, 1H, 16-CH), 4.51 (bs, 1H, 3-CH), 5.28 (s, 1H, 6-CH), 5.96 (dd, 1H, 26-CH, J = 15.6 Hz and 7.8 Hz), 6.26 (d, 1H, 28-CH, J = 15.6 Hz), 7.12 (m, 5H, aromatic protons of phenyl ring). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.83, 19.39, 19.72, 21.06, 21.79, 28.16, 30.06, 31.85, 31.99, 32.40, 32.66, 34.48, 37.12, 37.41, 38.04, 38.31, 38.51, 39.82, 41.10, 50.44, 57.31, 65.60, 74.33, 83.58, 90.82, 122.77, 126.39, 127.15, 128.73, 128.83, 137.05, 138.33, 140.09, 170.97; ESI mass (MeOH): 531.5 $[M+H]^+$, 553.5 $[M+K]^+$, 569.6 $[M+K]^+$.

Analogue **13** *(z) 26-(4′-nitrobenzylidene)-furost-5en-3β-acetate*: Yield = 62%, oil; ^{1}H NMR ($CDCl_3$): δ 0.71 (s, 3H, 18-CH_3), 0.96 (s, 3H, 19-CH_3), 1.05–1.88 (m, 26H, rest of the 2×CH_3, 7×CH_2 and 6×CH of steroidal ring), 2.02 (s, 3H, CH_3COO, acetate), 2.21 (bs, 2H, 4-CH_2), 2.30 (bd, 2H, 7-CH_2), 3.20 (bd, 1H, 22-CH), 4.20 (bs, 1H, 16-CH), 4.50 (bs, 1H, 3-CH), 5.26 (bs, 1H, 6-CH), 6.29 (m, 1H, 26-CH), 6.79 (d, 1H, 28-CH, J = 9.0 Hz), 7.33–8.05 (m, 4H, aromatic protons of phenyl ring). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.80, 19.29, 19.69, 20.62, 21.01, 21.81, 28.11, 29.74, 31.38, 31.95, 32.35, 32.99, 34.01, 37.09, 37.36, 38.22, 38.45, 39.74, 41.10, 50.37, 57.27, 65.40, 74.50, 83.69, 90.54, 122.77, 123.92, 124.33, 126.80, 130.53, 134.03, 140.04, 142.28, 143.26,162.83, 171.85; ESI mass (MeOH): 576.6 $[M+H]^+$, 574.6 $[M-H]^+$, 598.6 $[M+Na]^+$, 614.5 $[M+K]^+$.

Analogue **14** *(z) 26-(3′,4′,5′-trimethoxybenzylidene)-furost-5en-3β-acetate*: Yield = 29%, oil; ^{1}H NMR ($CDCl_3$): δ 0.79 (s, 3H, 18-CH_3), 0.95 (s, 3H, 19-CH_3), 1.04–1.87 (m, 29H, rest of the 2×CH_3, 8×CH_2 and 7×CH of steroidal ring), 2.02 (s, 3H, CH_3COO, acetate), 2.29 (bd, 2H, 7-CH_2), 3.85 (s, 9H, 3×OCH_3), 4.28 (bd, 1H, 22-CH), 4.61 (bs, 1H, 3-CH), 5.39 (t, 1H, 6-CH), 6.28 (d, 1H, 27-CH, J = 11.4 Hz), 6.32 (bd, 1H, 26-CH), 6.50 (d, 2H, 2′ and 6′-CH of phenyl ring). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.83, 19.41, 19.71, 21.03, 21.82, 28.14, 30.06, 31.97, 32.37, 32.61, 33.39, 35.13, 37.10, 37.39, 38.29, 38.48, 39.77, 41.06, 50.38, 56.42, 57.28, 61.30, 65.51, 74.30, 83.58, 90.71, 106.17, 122.77, 128.05, 133.94, 139.38, 140.09, 153.27, 170.97; ESI mass (MeOH): 621.5 $[M+H]^+$, 643.4 $[M+K]^+$, 659.4 $[M+K]^+$.

Analogue **15** *(E) 26-(3′,4′,5′-trimethoxybenzylidene)-furost-5en-3β-acetate*: Yield = 52%, oil; ^{1}H NMR ($CDCl_3$): δ 0.77 (s, 3H, 18-CH_3), 0.94 (s, 3H, 19-CH_3), 1.04–1.84 (m, 30H, rest of the 2×CH_3, 9×CH_2 and 6×CH of steroidal ring), 1.99 (s, 3H, CH_3COO, acetate), 2.29 (bd, 2H, 7-CH_2), 3.84 (s, 9H, 3×OCH_3), 4.10 (bd, 1H, 22-CH, J = 7.1 Hz), 4.27 (bs, 1H, 16-CH), 4.54 (bs, 1H, 3-CH), 5.33 (s, 1H, 6-CH), 5.98 (dd, 1H, 26-CH, J = 15.6 Hz and 7.8 Hz), 6.22 (d, 1H, 27-CH, J = 15.9 Hz), 6.54 (s, 2H, 2′ and 6′-CH of phenyl ring). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.83, 19.41, 19.70, 21.01, 21.77, 28.12, 30.07, 31.81, 31.96, 32.36, 32.65, 34.45, 37.08, 37.37, 37.96, 38.27, 38.47, 39.77, 41.06, 50.37, 56.41, 57.26, 61.26, 65.55, 74.24, 83.55, 90.72, 103.39, 122.74, 128.64, 134.05, 136.50, 137.61, 140.06, 153.63, 170.85; ESI mass (MeOH): 621.5 $[M+H]^+$, 643.5 $[M+Na]^+$, 659.4 $[M+K]^+$.

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2.2.6. General synthesis of aldoxime **16**

Analogue **16** *Furost-5-en-3β-acetoxy 26-aldoxime*: Yield = 70%, mp = 130–133 °C; ^{1}H NMR ($CDCl_3$): δ 0.78 (s, 3H, 18-CH_3), 0.97 (s, 3H, 19-CH_3), 1.07–1.86 (m, 26H, rest of the 2×CH_3, 8×CH_2 and 4×CH of steroidal ring), 2.01 (s, 3H, CH_3COO, acetate), 2.31 (bd, 2H, 7-CH_2), 3.29 (bs, 1H, 22-CH), 4.29 (bs, 1H, 3-CH), 4.59 (bd, 1H, 16-CH), 5.36 (s, 1H, 6-CH), 7.29 (bd, 1H, 26-CH, *J* = 6.3 Hz). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.81, 18.44, 19.26, 19.70, 21.03, 21.78, 28.12, 30.06, 31.32, 31.95, 32.36, 32.56, 35.06, 37.08, 37.38, 38.24, 38.47, 39.78, 41.07, 50.39, 57.28, 65.46, 74.32, 83.62, 90.39, 122.75, 140.06, 156.46, 170.01; ESI mass (MeOH): 472.4 $[M+H]^+$, 494.5 $[M+Na]^+$, 510.3 $[M+K]^+$.

2.3. Pharmacology

2.3.1. Cell culture

Primary cell culture was carried out as described previously [21]. In brief, the macrophage cells were collected 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 euthanised by cervical dislocation under ether anaesthesia and peritoneal macrophages were obtained by intra-peritoneal injection of phosphate buffer saline (PBS; pH 7.4). Membrane debris was removed by filtering the cell suspensions through sterile gauze. The viability of the 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 tissue culture plates in DMEM (Dulbecco modified Eagle medium, Sigma) supplemented with 10% foetal bovine serum with 1× stabilised antibiotic–antimycotic solution (Sigma) in a CO_2 incubator at 37 °C with 5% CO_2 and 90% relative humidity.

2.3.2. Quantification of pro-inflammatory cytokines

Cells were pretreated with 1 and 10 μg/mL of diosgenin analogues and standard anti-inflammatory drug dexamethasone (Sigma, USA), respectively, for 30 min. The cells were stimulated with lipopolysaccharide (LPS, *E. coli* 055:B5 Sigma, USA; 1 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 per well) with specific mouse TNF-α, IL-1β and IL-6 capture antibody 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) was added into each well. The plates were sealed and incubated for 1 h at RT and then washed thoroughly 5 times with wash buffer. 100 μL of tetramethylbenzidine (TMB) substrate solution was added to each well and the plate was incubated (without plate sealer) for 30 min at room temperature in the dark. Finally, 50 μL of stop solution (2 N H_2SO_4) was added to each well. The colour density was measured at 450 and 570 nm using a microplate reader (Spectramax; Molecular Devices, USA). Subtracted absorbance was measured at 570 nm from absorbance 450 nm. The values of TNF-α, IL-1β and IL-6 were expressed as pg/mL. The percentage (%) inhibition of pro-inflammatory cytokine production was calculated as follows:$\%\text{inhibition} = 100 \times \frac{(\text{concentration of vehicle control} - \text{concentration of test treatment})}{\text{OD of concentration of vehicle control}}$ where vehicle control indicates cells treated with vehicle in LPS-induced inflammation.

2.3.3. Cytotoxicity evaluation

Effect of diosgenin analogues on cytotoxicity was carried out in peritoneal macrophage cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. Peritoneal macrophage cells (0.5×10^6 cells/well) isolated from mice were suspended in DMEM medium (Sigma, USA) containing 10% heat-inactivated foetal bovine serum (Gibco, USA) and incubated in a 96-well culture plate at 37 °C in 5% CO_2 in an incubator and left overnight to attach. Cells were treated (1, 10 and 30 μg/mL) and incubated for 24 h at 37 °C in 5% CO_2. After treating the incubated cells, 20 μL aliquots of MTT solution (5 mg/mL in PBS) were added to each well and left for 4 h . Then, the MTT-containing medium was carefully removed and the formazan crystals formed were solubilised in DMSO (100 μL) for 10 min. The culture plate was placed on a micro-plate reader (Spectramax; Molecular Devices, USA) and the absorbance was measured at 550 nm. The amount of colour produced is directly proportional to the number of viable cells. Cell cytotoxicity was calculated as the percentage of MTT absorption as percentage (%) of survival = (mean experimental absorbance/mean control absorbance × 100).

2.3.4. In-vivo study

Animal experiments were carried out as per the approved protocol by the Institutional Animal Ethics Committee (IAEC) followed by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration no.: 400/01/AB/CPCSEA).

2.3.4.1. LPS-induced sepsis model in mice. BALB/c mice, 6–8 weeks of age, and weighing 18–22 g, were procured from the institute's animal house and acclimatised to the animal room for a week prior to experiment. The mice were fed with the standard mice feed and *ad libitum* drinking water under standard environmental conditions of 22 ± 3 °C, 12:12 dark-to-light cycle. After 1 week of adaptation, the mice were randomly divided into six groups of six mice each. Sepsis was induced in the experimental mice except normal group of mice by intra-peritoneal (i.p.) injection of LPS (5 mg/kg) in normal saline. Analogue **15** was dissolved in 0.7% carboxymethyl cellulose (CMC) to obtain a uniform suspension and administered orally 24 h and 2 h before LPS injection.

Group 1: Normal control administered CMC
Group 2: Vehicle control administered CMC + LPS
Group 3: Analogue **15** (3 mg/kg) + LPS
Group 4: Analogue **15** (10 mg/kg) + LPS
Group 5: Analogue **15** (30 mg/kg) + LPS

Blood was collected 2 h after LPS administration from orbital plexus and serum was separated and stored at −80 °C until analysis. Pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) from serum were quantified using commercially available mouse-specific enzyme immune assay kits (BD Biosciences, USA) by following the manufacturer's instruction.

2.3.4.2. Histopathology examination. Liver and lung samples were obtained 24 h after LPS challenge. Tissues were collected and fixed in 10% buffered formalin. After fixation, tissues were rinsed with water, dehydrated with graded concentration of ethanol and embedded in paraffin wax. The samples were sectioned into 5 μm thick and mounted on glass slides. The sections were then dewaxed using xylene and ethanol, and stained with haematoxylin and eosin (H&E stain). A representative area was selected for qualitative light microscopic analysis under 100× magnification.

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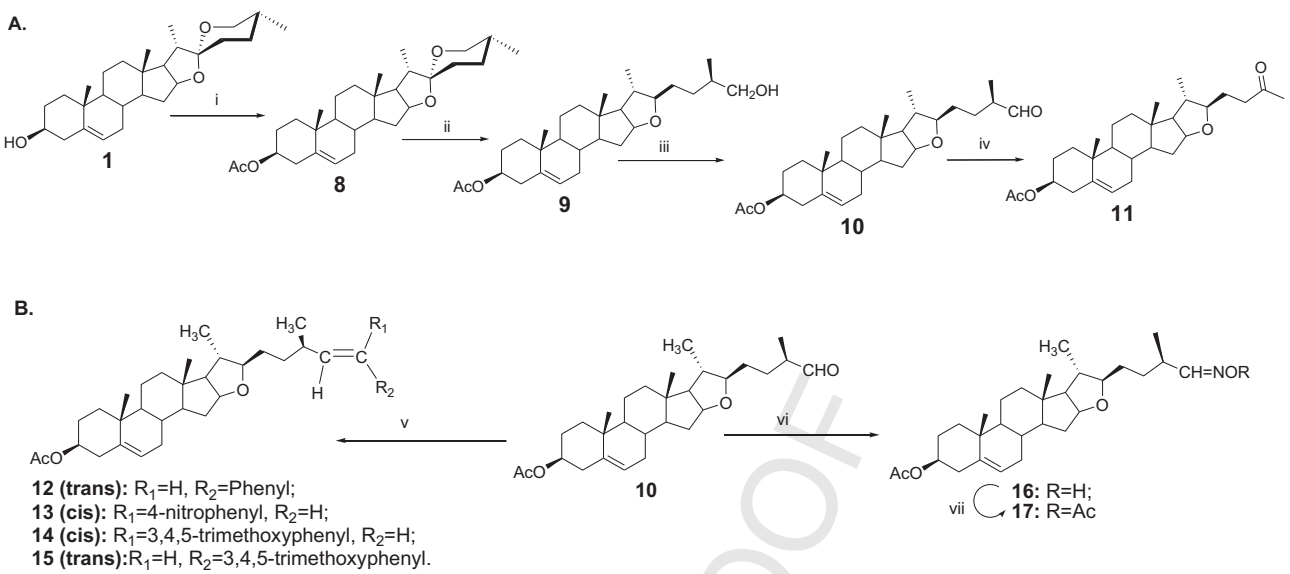

Scheme 1. (i) Ac_2O, dry pyridine, $CHCl_3$, RT, 2 h, 91%; (ii) AcOH, $NaCNBH_4$, RT, 3 h, 81%; (iii) dry DCM, PCC, reflux, 1 h, 91%; (iv) ethanol, 3,4,5-trimethoxyaniline, RT, 2 h, 84%; (v) Wittig salt, NaH, toluene, reflux, 3–4 h, 29–68%; (vi) $NH_2OH{\cdot}HCl$, EtOH, reflux, 2 h, 70%; (vii) Ac_2O, dry pyridine, dry $CHCl_3$, RT, 92%.

2.3.4.3. Survival study. The second set of experiments was performed to examine the effect of analogue **15** on LPS-induced lethality in mice. The female BALB/c mice were divided into five groups ($n = 6$). The treatment group received analogue **15** orally at doses of 3, 10 and 30 mg/kg, 24 and 2 h before injection of LPS, the LPS group received an intraperitoneal injection at a dose of 15 mg/kg, and the control group received the equal amount of vehicle instead of analogue **15**. Survival of mice was monitored every 12 h for 5 days.

2.3.5. Acute oral toxicity study

Acute oral toxicity study of the analogue **15** was done on BALB/c male mice as described previously [22]. For this study, mice administered orally with analogue **15** (300 mg/kg body weight) were considered as the test group and mice treated with corresponding volume of vehicle (0.7% carboxymethyl cellulose [CMC]) were considered the control group. Mice were observed individually, after dosing, at least once during the first 30 min, periodically during the first 24 h and daily thereafter for a total of 7 days.

2.4. Molecular docking

Two-dimensional molecular structures were drawn with the ChemDraw Ultra and energy minimization was performed with MM2/MM3 molecular mechanics parameter until achieving the lowest stable energy state. This energy minimization process was performed until the energy change was less than 0.001 kcal mol^{-1} or the molecules had been updated almost 300 times [23]. The 3D chemical structure of known drug was collected from the PubChem compound database of NCBI, USA (http://www.pubchem.ncbi.nlm.nih.gov). Crystallographic 3D structures of target proteins TNF-α (PDB: 2AZ5), IL-6 (PDB: 1ALU) and IL-1β (PDB: 9ILB) were retrieved from the Brookhaven Protein Databank (PDB) (http://www.pdb.org). Hydrogen atoms were added to the protein targets to achieve the correct ionisation and tautomeric states of amino acid residues such as His, Asp, Ser and Glu. Molecular docking of the compounds against selected target was achieved using the 'AutoDock Vina' software. To perform the automated docking of ligands into the active sites, we used a Lamarckian genetic algorithm [24].

2.5. Statistical analysis

Results were presented as the means ± SE and analysed using GraphPad Prism 4. The ANOVA followed by Tukey's multiple comparison test was used to assess the statistical significance of vehicle vs treatment groups. Results are presented as the means ± SE. Differences with a p value < 0.05 were considered significant.

3. Results

3.1. Chemistry

The synthetic strategy was depicted as Scheme 1. Diosgenin (**1**) was acetylated with acetic anhydride–pyridine system in dry chloroform to get diosgenin 3-acetate (**8**) at room temperature. The spiroketal bond was reduced with sodium cyanoborohydride in acetic acid to get a primary alcohol (**9**). The alcohol **9** was oxidised to its corresponding aldehyde (**10**) by treating it with pyridinium chlorochromate (PCC) in dichloromethane.

Further, while preparing Schiff's bases of aldehyde **10** with an aromatic amine, ketone **11** was obtained unexpectedly. Compound **10** was reacted with an aromatic amine (aniline/3,4-methylenedioxyaniline/3,4,5-trimethoxyaniline) in ethanol and each time it was transformed to ketone **11**. We did not do any mechanistic study to establish this transformation. However, it is assumed that after the formation of a Schiff's base, it was converted to an enamine. Further decomposition of enamine by water might have converted it to ketone **11**. The structure of **11** was established by 1D, 2D NMR, ESI-HRMS and finally by X-ray crystallography [25].

Diverse analogues were prepared on aldehyde **10**. Three different Wittig salts (benzyltriphenylphosphonium bromide, 4-nitrobenzyltriphenylphosphonium bromide, 3,4,5-trimethoxybenzyltriphenylphosphonium bromide) were treated with sodium hydride–toluene system to get styrene type analogues **(12–15)** at C27 of steroidal framework of aldehyde **10**. Aldoxime (**16**) and oxime-acetate (**17**) were also prepared on **10** by treating with hydroxylamine hydrochloride and then acetylating. All theses diosgenin analogues were confirmed through spectroscopy.

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Table 1
Q11 Effect of diosgenin analogues on production of pro-inflammatory cytokines in LPS-induced inflammation in macrophage cells..

Analogues	Dose (μg/mL)	Pro-inflammatory cytokine (pg/mL)		
		TNF-α	IL-6	IL-1β
Basal	–	68.17 ± 9.67	93.52 ± 5.14	22.65 ± 4.09
Vehicle	–	1301.07 ± 93.68#	912.63 ± 21.71#	103.08 ± 8.72#
Diosgenin				
1	1	1138.74 ± 77.05ns	792.58 ± 49.25ns	96.50 ± 3.18ns
	10	1052.72 ± 93.68*	693.69 ± 16.10*	81.33 ± 7.23ns
8	1	1192.86 ± 79.42ns	777.13 ± 15.17ns	98.00 ± 6.18ns
	10	794.84 ± 13.09*	718.24 ± 19.70*	87.33 ± 4.98ns
9	1	1077.04 ± 66.6ns	841.72 ± 61.28ns	92.46 ± 7.47ns
	10	801.94 ± 53.08*	774.53 ± 61.50ns	82.46 ± 6.06ns
10	1	1037.36 ± 43.22ns	822.39 ± 30.99ns	98.67 ± 6.94ns
	10	810.96 ± 24.37*	781.33 ± 34.57ns	93.45 ± 4.09ns
11	1	1227.5 ± 58.02ns	740.96 ± 57.40ns	88.67 ± 10.27ns
	10	1155.38 ± 45.14ns	727.53 ± 52.43*	83.61 ± 6.13ns
12	1	1049.54 ± 73.35ns	599.58 ± 13.33*	98.60 ± 4.70ns
	10	548.76 ± 58.51*	569.58 ± 32.86*	82.67 ± 8.42ns
13	1	1013.42 ± 46.32*	551.97 ± 27.30*	98.65 ± 4.45ns
	10	623.78 ± 63.25*	540.14 ± 33.92*	95.33 ± 7.54ns
14	1	971.20 ± 75.12*	557.82 ± 16.70*	97.00 ± 1.77ns
	10	458.94 ± 55.30*	465.60 ± 12.43*	84.67 ± 2.34ns
15	1	950.220 ± 33.90*	517.61 ± 25.54*	93.50 ± 5.18ns
	10	307.56 ± 65.03*	387.61 ± 16.01*	69.00 ± 8.15*
16	1	1215.66 ± 54.18ns	758.15 ± 52.4ns	96.0 ± 6.24ns
	10	1178.92 ± 61.79ns	687.64 ± 34.12*	94.44 ± 5.26ns
17	1	1067.20 ± 63.58ns	667.26 ± 57.24*	92.50 ± 7.23ns
	10	493.38 ± 45.40*	591.70 ± 16.89*	70.67 ± 8.02*
Dexamethasone	1	439.48 ± 57.49*	313.85 ± 50.57*	88.50 ± 2.73ns
	10	177.04 ± 35.18*	163.74 ± 10.18*	57.66 ± 8.85*

$n = 3$.
Normal vs vehicle.
* $p < 0.05$, vehicle vs treatment.

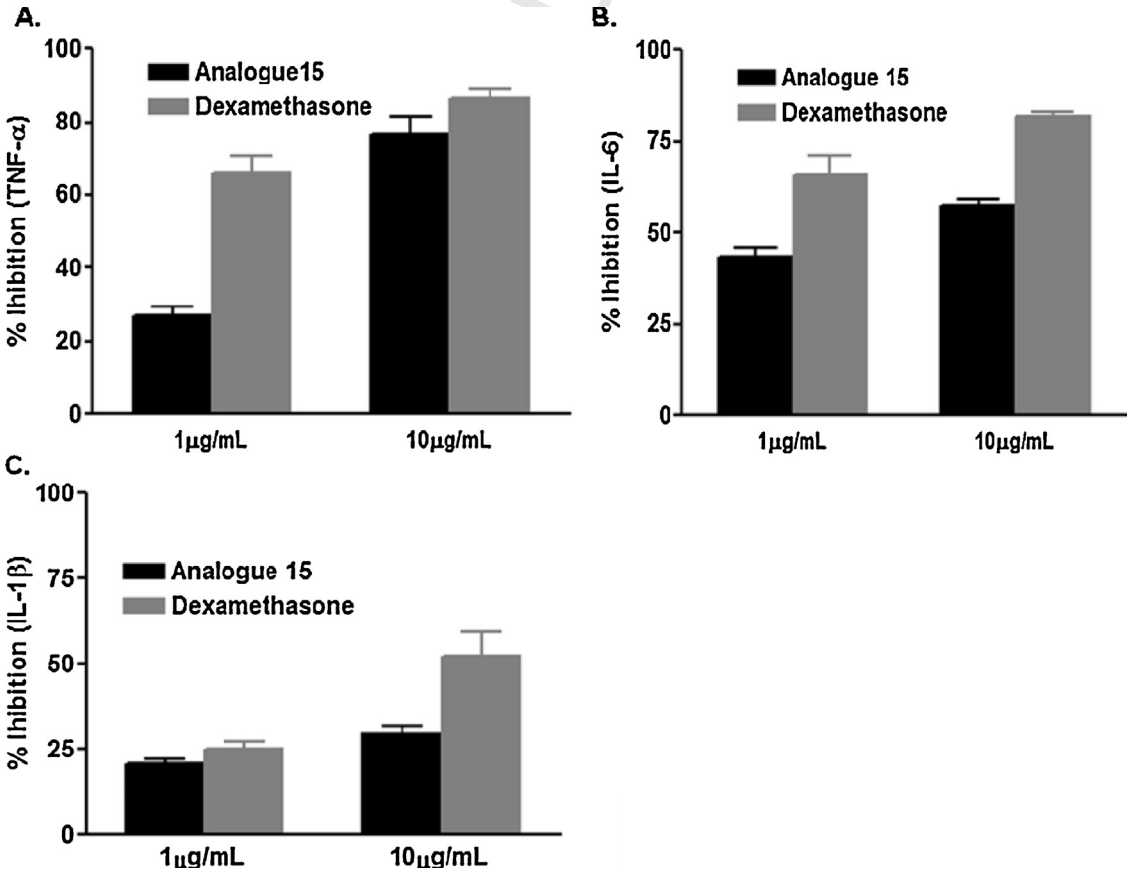

Fig. 3. Effect of analogue **15** and dexamathasone on percent (%) inhibition of pro-inflammatory cytokine production in LPS-induced inflammation in macrophage cells. (A) TNF-α; (B) IL-6; (C) IL-1β.

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3.2. Pharmacology

3.2.1. Effect of diosgenin analogues on LPS-induced pro-inflammatory cytokines production in macrophages

All the synthesised diosgenin analogues were evaluated for their anti-inflammatory status against the production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) using ELISA technique in LPS-induced inflammation in macrophage cells at the concentrations of 1 and 10 μg/mL.

Production of pro-inflammatory cytokines was significantly ($p < 0.05$) increased in LPS-stimulated cells when compared with normal un-stimulated cells. All the analogues including parent diosgenin exhibited inhibition of pro-inflammatory cytokines production when compared with vehicle treated LPS-stimulated cells (Table 1).

Among the all analogues, analogue **15** [(E) 26-(3′,4′,5′-trimethoxybenzylidene)-furost-5en-3β-acetate] possessed significant inhibition of TNF-α, IL-1β and IL-6 at both the concentration (Table 1). The percent inhibition of pro-inflammatory cytokines by analogue **15** in comparison to dexamethasone, a standard anti-inflammatory steroid drug is depicted in Fig. 3. These pro-inflammatory cytokines are the mediators of various acute and chronic inflammation linked diseases. Based on this study, analogue **15** was chosen for its further validation for evaluation of efficacy and safety in *in-vivo* system using LPS-induced sepsis as a systemic inflammation model and acute oral toxicity respectively in mice.

3.2.2. In-vitro cytotoxicity profile of diosgenin analogues

We first examined the *in-vitro* cytotoxicity profile of all synthesised diosgenin analogues using MTT assay in peritoneal macrophage cells isolated from mice. The significant change in percent live cell population was not observed ($p < 0.05$) at any concentration of diosgenin analogues treatment when compared with normal cells. Results are summarised in Table 2.

Table 2
Effect of diosgenin analogues on percent (%) cell viability in macrophage cells using MTT assay.

Analogues	Dose (μg/mL)	% Cell viability
Diosgenin		
1	1	104.31 ± 5.97
	10	101.18 ± 1.97
	30	97.73 ± 1.78
8	1	104.25 ± 9.05
	10	99.62 ± 1.77
	30	97.80 ± 3.08
9	1	99.42 ± 2.66
	10	96.87 ± 3.71
	30	93.34 ± 1.39
10	1	105.40 ± 2.41
	10	101.81 ± 4.85
	30	95.17 ± 5.81
11	1	102.22 ± 2.66
	10	99.63 ± 4.48
	30	94.47 ± 6.09
12	1	101.62 ± 3.56
	10	97.92 ± 2.64
	30	96.62 ± 5.34
13	1	100.25 ± 2.87
	10	98.20 ± 0.90
	30	97.13 ± 1.18
14	1	101.33 ± 3.55
	10	99.65 ± 1.96
	30	98.25 ± 2.78
15	1	103.28 ± 6.28
	10	100.78 ± 2.67
	30	96.73 ± 2.11
16	1	104.21 ± 4.19
	10	100.94 ± 3.67
	30	94.51 ± 4.40
17	1	102.83 ± 3.30
	10	97.26 ± 2.69
	30	97.57 ± 4.17
Dexamethasone		
	1	99.87 ± 3.92
	10	96.11 ± 5.55
	30	93.78 ± 8.71

$n = 3$.

3.2.3. Effect of analogue **15** on pro-inflammatory cytokine production in sepsis model of mice

To substantiate the physiological function of analogue **15** in *in-vivo* system, we examine the therapeutic anti-inflammatory effect of analogue **15** in a mouse model of sepsis, a systemic inflammatory condition. Serum level of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) was significantly increased in LPS-challenged vehicle-treated mice when compared to normal mice. Oral administration of analogue **15** before LPS challenge significantly ($p < 0.05$) inhibited TNF-α and IL-6 production in a dose-dependent manner at dose rates of 3, 10 and 30 mg/kg body weight. The higher pretreatment dose (10 and 30 mg/kg) could also inhibit the IL-1β production in serum (Fig. 4).

3.2.4. Histopathological changes in lung and liver tissues

To elucidate the effect of analogue **15** on lung and liver injuries in sepsis model of mice, histopathological examination of liver and lung tissue section was performed. Significant damage was observed in liver and lung tissues as evidenced by inflammatory cell infiltration in LPS-challenged vehicle-treated mice when compared with normal mice. Pretreatment of analogue **15** ameliorated the tissue damage in a dose-dependent manner (Fig. 5).

3.2.5. Effect of analogue **15** on LPS-induced mortality in mice

The survival study was conducted to assess the protective effect of analogue **15** on LPS-induced mortality in mice. We monitored its effect on survival of mice for 5 days at 12 h. All the mice in LPS-challenged vehicle-treated group died within 48 h. Pretreatment of analogue **15** resulted in a markedly improved survival in dose-dependent manner at 5 days and these animals are still survived (Fig. 6).

3.3. Acute oral toxicity study

The acute toxicity study showed that a single oral administration of analogue **15** (300 mg/kg) did not produce any mortality, behavioural changes (gait, posture, fur, depression and panting) in the mice as compared to the control group. Similarly, no significant changes were recorded in body weight, organ weight, serum biochemical (total bilirubin, creatinine, triglycerides, SGOT and glucose) as well as haematology parameters (total RBCs and total WBCs) of the treated group when compared to the control group. The representative results are depicted in Table 3 and Fig. 7.

3.3.1. Molecular interaction study of diosgenin analogues through docking

In-vitro and *in-vivo* anti-inflammatory profiles of diosgenin analogues were further confirmed by molecular docking experiments. The aim of the molecular interaction study was to explore the molecular interaction of diosgenin analogues with pro-inflammatory cytokine receptors. The interaction study was compared with dexamethasone, a standard steroidal anti-inflammatory drug (Table 4).

The studied molecules show molecular interaction with TNF-α (PDB: 2AZ5), IL-6 (PDB: 1ALU) and IL-1β (PDB: 9ILB). The representative molecular interaction results of analogue **15** and dexamethasone are depicted in Fig. 8.

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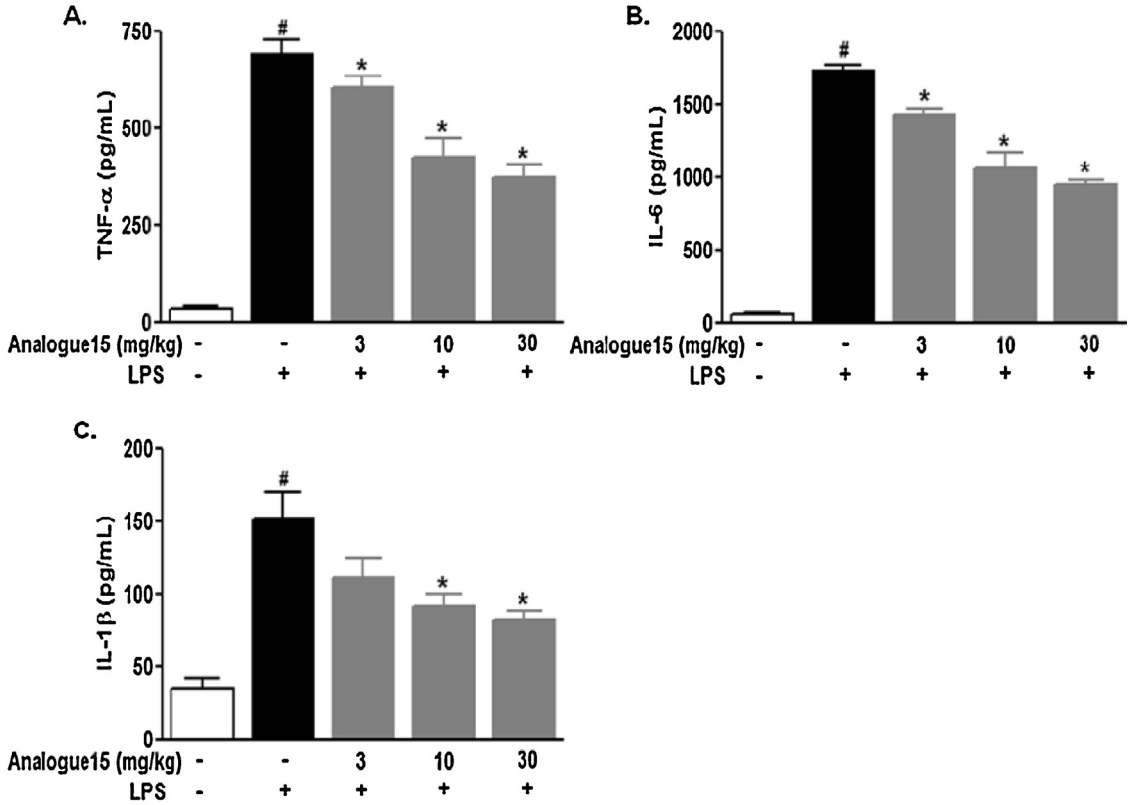

Fig. 4. Effect of analogue **15** on pro-inflammatory cytokine production in sepsis model of mice. (A) TNF-α; (B) IL-6; (C) IL-1β. Data are expressed as mean ± SEM: $^{*}p < 0.05$; vehicle vs treatment; # vehicle vs normal; (Tukey's multiple comparison test); $n = 6$.

TNF-α interaction study found that residues Tyr-119, Leu-120, Gly-121, Leu-57, Tyr-59, Ser-60, Gly-122 and Tyr-151 were commonly shared by dexamethasone and diosgenin analogue **15**. Similarly analysis of binding pocket residues of IL-6 interacted with the diosgenin analogue **15**, and dexamethasone shared Asn-63, Leu-64, Pro-65, Glu-93, Val-96, Tyr-97, Pro-139, Thr-143, Asn-144, Leu-147 and Lys-150 residues. IL-1β interaction with the diosgenin analogue **15** and dexamethasone revealed the common residues as Leu-57, Ile-58, Tyr-59, Ser-60, Gln-61, Asn-63, Leu-64, Pro-65, Gly-122, Tyr-151, Ile-155, Tyr-119, Leu-120 and Gly-121.

4. Discussion

There has been increasing interest in the discovery and development of novel pharmaceuticals from natural origin that have the same or better efficacy accompanied by less side effects. The majority of natural molecules and their analogues showed potent anti-inflammatory activities and some established themselves as clinical agents for chronic inflammatory disease conditions. Therefore, plant-based natural compounds play a significant role in the development of anti-inflammatory drugs in the pharmaceutical industry which can serve as good lead molecules suitable

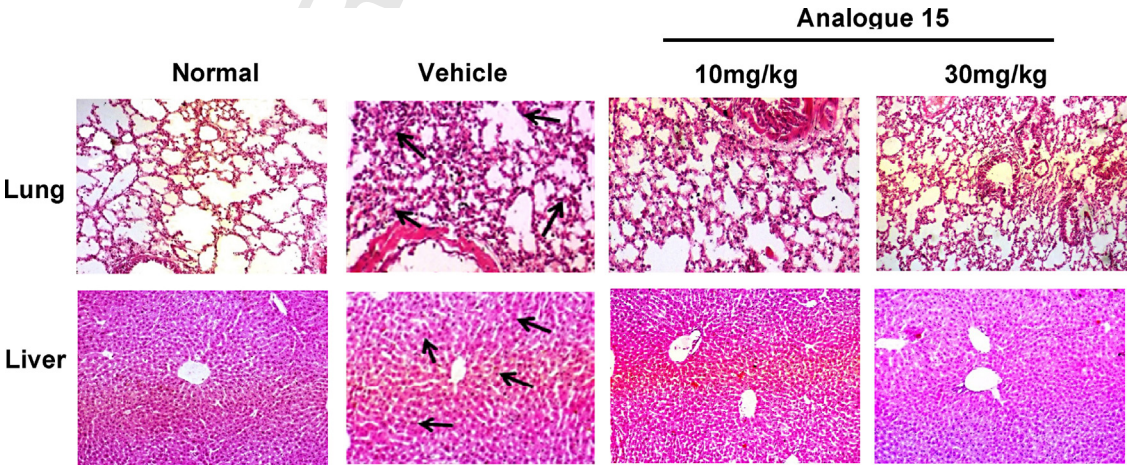

Fig. 5. Representative microphotograph of lung and liver (H&E stain, 100×) sections from the mice. Arrows indicate leukocyte infiltration.

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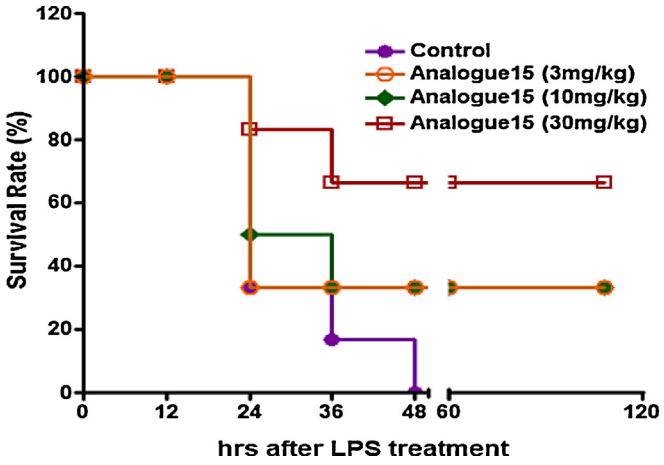

Fig. 6. Effect of analogue **15** on LPS-induced mortality in mice.

for further modification during the drug development process. Diosgenin (DG) is a C27 spiroketal steroidal sapogenin abundantly available in nature. It is mainly present in the form of saponin in the plants [26]. Previous investigations have shown that diosgenin plays an important pharmacological role including anti-inflammatory activity [18]. In present study, we have reported the influence of synthesised analogues of diosgenin by modifying at spiroketal ring on LPS-induced inflammatory response; an *in-vitro*, *in-vivo* study and it was further confirmed using *in-silico* study.

We have evaluated the *in-vitro* anti-inflammatory status of the synthesised diosgenin analogues against the production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β). Synthesised analogues along with parent diosgenin exhibited inhibition of pro-inflammatory cytokines productions in LPS-stimulated cells without any cytotoxic effect as assessed by MTT assay. Several previous studies reported that diosgenin, a parent molecule, reduces the production of inflammatory mediators by inhibiting CK2, JNK, NF-κB and AP-1 activation [18,27]. This study, to our knowledge, provides the first evidence that synthesised analogues of diosgenin modifies at spiroketal ring exhibiting anti-inflammatory activity by inhibiting the production of pro-inflammatory cytokines in LPS-induced inflammation in macrophage cells. Pro-inflammatory cytokines, namely, TNF-α, IL-1β and IL-6, in LPS-induced macrophages, are known to have profound effects on the regulation of immune reactions, haematopoiesis and inflammation [28]. Overproduction of these cytokines has been implicated in the pathogenesis of many disease processes. The control of macrophage overproduction of these mediators should greatly facilitate the treatment of many inflammation-linked diseases such as sepsis, rheumatoid arthritis and autoimmune diabetes [16,17].

Among all analogues, analogue **15** [(E) 26-(3′,4′,5′-trimethoxybenzylidene)-furost-5en-3β-acetate] possessed significant ($p < 0.05$) inhibition of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) at both the concentrations. It would also be interesting to evaluate its therapeutic effect in *in-vivo* systemic inflammation model. To substantiate the physiological function of most potent analogue, we have further evaluated the therapeutic efficacy and safety profile of analogue **15** in *in-vivo* system using LPS-induced sepsis and acute oral toxicity respectively in mice. Sepsis is marked by a systemic inflammatory response. Persistent or inappropriate or overproduction of multiple pro-inflammatory mediators such as TNF-α and IL-6 leads to severe injury and

Table 3
Effect of analogue **15** on acute toxicity at 300 mg/kg as a single oral dose in BALB/c mice.

Parameters studied	0th day		7th day	
	Control	Experimental	Control	Experimental
Body weight (g)	21.85 ± 0.36	21.80 ± 0.40	22.53 ± 0.49	22.6 ± 0.361
SGOT (U/L)	32.30 ± 4.34	31.56 ± 3.21	31.50 ± 5.34	31.06 ± 8.42
SGPT (U/L)	23.46 ± 4.01	22.45 ± 3.02	23.83 ± 7.53	21.96 ± 3.28
Cholesterol (mg/dL)	101.03 ± 1.33	105 ± 2.87	103.0 ± 1.69	100.28 ± 1.58
Triglycerides (mg/dL)	147.09 ± 7.28	128.47 ± 5.71	106.93 ± 7.14	108.69 ± 4.67
Haemoglobin (g/dL)	14.81 ± 2.11	14.96 ± 1.34	13.42 ± 1.51	14.44 ± 0.86
RBC (million/mm^3)	7.31 ± 1.17	7.80 ± 1.33	7.58 ± 0.38	7.21 ± 0.41
WBC (thousands/mm^3)	4.09 ± 0.12	4.35 ± 0.75	5.51 ± 0.19	4.32 ± 0.34

Data are expressed as mean ± S.E.M., $n = 6$.

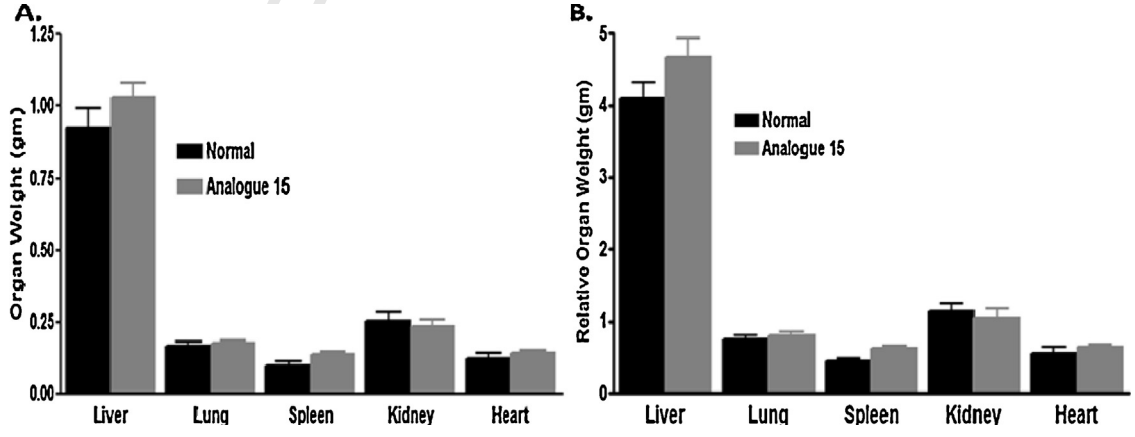

Fig. 7. Effect of analogue **15** as a single acute oral dose at 300 mg/kg on (A) absolute and (B) relative organ weight in BALB/c albino mice ($n = 6$, non significant changes were found compared to control).

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Table 4
Binding affinity (kcal mol^{-1}) and interacting residual information of diosgenin derivatives with respect to the anti-inflammatory targets TNF-α (PDB: 2AZ5), IL-6 (PDB: 1ALU) and IL-1β (PDB: 9ILB).

Protein	Ligands	Binding affinity (kcal mol^{-1})	Pocket residues
TNF-α (2AZ5)	Analogue **15**	−9	Gln(C)-61, Gly(C)-121, Gly(C)-122, Ile(C)-58, Ile(C)-155, Leu(C)-57, Leu(C)-120, Ser(C)-60, Tyr(C)-59, Tyr(C)-119, Tyr(C)-151, Gly(D)-121, Leu(D)-57, Leu(D)-120, Ser(D)-60, Tyr(D)-59, Tyr(D)-119, Tyr(D)-151
	Dexamethasone	−7.9	Gln(B)-125, Leu(B)-55, Leu(B)-157, Gly(C)-121, Gly(C)-122, Leu(C)-57, Leu(C)-120, Tyr(C)-59, Tyr(C)-119, Gly(D)-121, Leu(D)-120, Ser(D)-60, Tyr(D)-59, Tyr(D)-119, Tyr(D)-151
IL-6 (1ALU)	Analogue **15**	−6	Asn-63, Leu-64, Pro-65, Lys-66, Lys-86, Glu-93, Val-96, Tyr-97, Pro-139, Asp-140, Thr-143, Asn-144, Leu-147, Lys-150
	Dexamethasone	−6.4	Asn-61, Asn-63, Asn-144, Glu-93, Leu-64, Leu-147, Lys-150, Pro-65, Pro-139, Thr-143, Tyr-97, Val-96
IL-1β (9ILB)	Analogue **15**	−5.7	Arg-4, Asn-108, Glu-51, Glu-105, Ile-56, Leu-6, Leu-110, Lys-103, Met-44, Met-148, Phe-46, Phe-150, Ser-5
	Dexamethasone	−7.4	Asn-7, Asn-66, Asp-86, Glu-64, Leu-62, Lys-63, Lys-65, Pro-87, Pro-91, Ser-43, Ser-153, Tyr-68, Tyr-90, Val-85

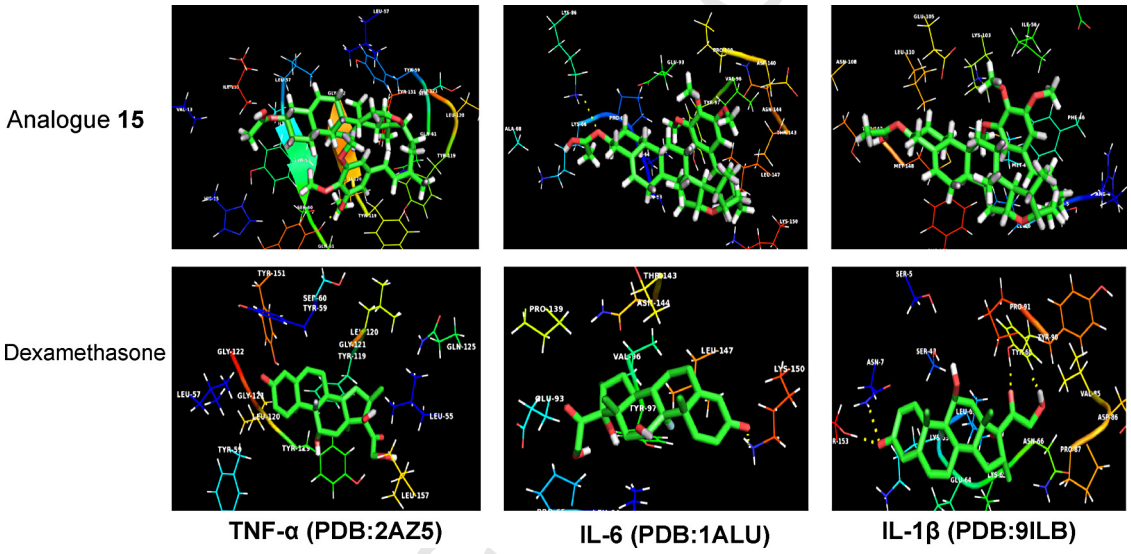

Fig. 8. Analogue **15** and dexamethasone docked on target protein; TNF-α (PDB: 2AZ5), IL-6 (PDB: 1ALU) and IL-1β (PDB: 9ILB).

increased mortality in sepsis [29,30]. Thus, regulation of multiple mediators could be more beneficial than suppression of single mediator. In fact, the clinical trials targeting single inflammatory cytokine have been proved ineffective in the treatment of sepsis [31,32]. In LPS-induced sepsis model study in mice, we demonstrated that the oral administration of analogue **15** before LPS challenge significantly ($p < 0.05$) inhibited TNF-α and IL-6 production in dose-dependent manner at the dose rate of 3, 10 and 30 mg/kg body weight and higher dose (30 mg/kg) is required to inhibit ($p < 0.05$) the IL-1β production in serum. Pretreatment of analogue **15** also ameliorated the lung and liver injuries in sepsis model of mice as an evidence of histopathological examination as well as it helps to improve the survival of mice in lethal sepsis model. Overproduction of TNF-α and IL-1β leads to tissue damage [33,34], multiple organ failure and finally causes lethal sepsis [21]. Therefore, agents attenuating the production of pro-inflammatory cytokines may have potential as treatments for prevention of lethal sepsis [22].

Molecular interaction study of diosgenin analogue **15** with pro-inflammatory targets (TNF-α, IL-6 and IL-1β) through docking showed high binding affinity i.e. low docking energy. Several previous reports also concluded that the molecules having high binding affinity with targeted protein exhibited therapeutic efficacy [35,36].

5. Conclusion

Collectively, we demonstrated that diosgenin analogues inhibit the production of pro-inflammatory cytokines in both *in-vitro* and *in-vivo* condition, it was further confirmed with docking study. This finding confirms the suitability of diosgenin analogues as candidates for further investigation towards the management of inflammation related diseases.

Conflict of interest

There are no conflicts of interest.

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