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Oxysterols: Synthesis and anti-leishmanial activities

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ABSTRACT

Oxygenated sterols (**2**–**16**) were synthesized by skeletal rearrangement of steroidal allylic alcohols. All the derivatives were screened for their anti-leishmanial activities. Compounds **3**, **11** and **12** showed potent activities. Compound **12** was found least toxic and induced highest nitric oxide (NO) at 48 h. Least toxicity of compound **12** on splenocytes validated its best anti-amastigote effect and induction of NO.

1. Introduction

Sterols are essential for life in mammals (as well as in lower eukaryotes such as yeast and fungi) and have a central role in numerous physiological processes. Oxysterols including both autoxidation products and sterol metabolites have many important biological activities mostly related to the physiological control of cholesterol biosynthesis [1]. 7α-Hydroxy-cholest-4-ene-3-one is an intermediate in the conversion of cholesterol into bile acids [2]. Some of the oxysterol derivatives might be used as markers to physiological efficiencies [2] and display many biological activities in normal and diseased states. Derivatives of cholesterol oxygenated in the 7, 20, 22 or 25 positions depress the activity of the regulatory enzyme in the sterol synthetic pathway, 3-hydroxy-3-methyl glutaryl-coenzyme (HMG CoA) reductase within 6 h [1,3]. Specific naturally occurring oxysterols have potent osteoinductive and anti-adipogenic properties [4,5]. Parhami and co-workers reported that some oxysterols analogs induced the osteogenic effects and inhibit the adipogenic differentiation of bone marrow stromal cells (MSC) through activation of Hedgehog (Hh) signaling [6]. Researchers have also reported the cytotoxicity [7–9], anti HIV [10] and anti-asthma [11] properties of various oxygenated sterol derivatives.

Leishmaniasis constitutes a group of human diseases caused by obligate intracellular protozoan parasites of genus *Leishmania*. It is the second most common parasitic disease in the modern world, behind malaria [12]. Leishmaniasis has a worldwide distribution with important foci of infection in Central and South America, Southern Europe, North and East Africa, the Middle East and the Indian subcontinent. In recent times the main foci of VL are in Sudan and India [13].

Today some antimony containing drugs are generally used to treat *Leishmania*. The toxicity of the available drugs and the emergence of strains that are not responsive to drug therapy make the discovery of novel therapeutic agents imperative [14]. Recently miltefosine (hexadecylphosphocholine or HPC), originally developed as an anticancer agent, has been introduced as an effective anti-leishmanial drug. However, miltefosine-associated gastrointestinal toxicity and teratogenicity have already been identified during clinical trials in India. The long half-life of the drug might also encourage the emergence of resistant lines, as evidenced from selected miltefosine resistant line of *Leishmania donovani* and *Leishmania tropica in vitro*, is also another matter of concern [15]. Moreover, cases indicating the relapse of the disease, even after 10 months of a full course of treatment with miltefosine [16] prompted researchers to search for novel molecules active against the *Leishmania* infection. Therefore, development of new chemotherapeutics with lower toxicity and higher efficiency is the high demand in contemporary medicinal and pharmaceutical sciences. In the present study synthesis of some oxygenated cholesterol derivatives **1**–**16** along with their anti-leishmanial activities were evaluated and are being reported here. On proliferation of HSCs-T6, compound **3** showed the prominent inhibitory activity and the LC50 value was 90.5 μg/mL [17]. Compound 12 was previously prepared from the 7-keto compound by using $NaBH_4/CeCl_3{\cdot}H_2O$ and it showed no significant antimicrobial activity [18].

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2. Experimental

Melting points were determined by the open capillary method and are uncorrected. The NMR spectra were recorded in $CDCl_3$ solutions at ambient temperature on a Bruker Avance 300 MHz NMR spectrometer using 5 mm BBO (i.e. broadband observe) probe. The chemical shifts δ are given in ppm related to tetramethylsilane (TMS). The coupling constants (*J*) are reported in Hz. IR spectra were recorded in Shimadzu 8300 FT-IR spectrophotometer in KBr discs. All solvents were purified according to reported procedures and reagents were used as commercially available. The mass spectra were taken in 4800 (ABSciex) MALDI-TOF/TOF Tandem Mass Spectrometer. Elemental Analyses were done in Vario EL-III.

2.1. Jone's Oxidation of Cholesterol **1**

Jone's Oxidation of Cholesterol was carried out following the previous reports [19] and Cholest-4(5)en-3-one 2 (47%) and Cholest-4(5)en-3,6-dione 3 (40%) were obtained. However, compound **3** was prepared earlier from the 5α,6β-diols by using N-bromosuccinimide [20].

2.2. Sodium borohydride reduction of Cholest-4(5)en-3,6-dione **3**

Cholest-4(5)en-3,6-dione (650 mg, 1.63 mmol) was dissolved in a mixture of dioxane and methanol (15 mL each) was added with cooling (0–5 °C) a slurry of sodium borohydride (20.45 mg, 0.54 mmol) prepared in an ammonium chloride-ammonium hydroxide buffer (pH = 8, 3 mL) and the mixture was stirred at room temperature for 3 h. Most of the solvents were removed by distillation, cooled and acidified with 1 M HCl and then extracted with ether (3 × 50 ml). The ethereal layer was washed with water till neutral and dried by anhydrous sodium sulfate and purified over a column of silica gel [60–120 mesh, 100 g, 25 cm, eluent 1% ethyl acetate (EA) in pet. ether (PE)].

2.2.1. Cholest-4(5)en-3-ol-6-one **5**

White solid; 35% yield; melting point (mp): 142–143 °C; 400.34 [M^+]; IR: 3386, 2841, 2669, 1716, 1652, 1508, 1462, 1377, 1168, 850, 723, 669 cm^{-1}; UV: 232 nm (3320); ^{1}H NMR: δ_H 0.71 (s, 3H, H_3-18), 1.12 (s, 3H, H_3-19), 2.40 (m, 2H, H_2-2); 3.21 (s, 1H, —OH at C_3), 4.31 (d, 1H, *J* = 9.3 Hz, H-3), 6.17 (s, 1H, H-4); ^{13}C NMR: δ_C 200.1, 172.7, 119.6, 68.4, 56, 55.5, 53.7, 42.4, 41.2, 39.4, 39, 36.1, 36, 35.7, 34.1, 33.7, 28.1, 27.9, 24.1, 23.8, 22.8, 22.5, 21.0, 18.6, 18.2, 11.9. Elemental analysis: $C_{27}H_{44}O_2$ requires C, 80.94%; H, 11.07%; O, 7.99%; found C, 80.98%; H, 11.01%, O, 7.92%.

2.2.2. Cholest-4(5)en-3,6-diol **6**

The observed spectroscopic analysis agreed well with the literature data [21].

2.3. Treatment of H_2O_2-p-TsOH on Cholest-4(5)en-3-ol-6-one **5**

To a solution of Cholest-4(5)en-3-ol-6-one (200 mg, 0.49 mmol) in methylene chloride (50 mL) was added 40 mL of oxidizing solution prepared from 3 mL 30% H_2O_2 in 80 mL of t-BuOH containing 3.0 g of *p*-toluene sulfonic acid at room temperature. The solution was stirred slowly for 72 h was then poured onto water. The mixture was extracted with methylene chloride (3 × 50 ml), washed with water, aqueous sodium bicarbonate solution and then again with water. The organic layer was dried off, evaporated and purification by column chromatography (silica gel 60–120 mesh, 50 g, 12 cm) yielded two compounds (eluent 1% EA in PE and 2% EA in PE).

2.3.1. Cholest-3(4)epoxy-6-one **7**

White solid; 48% yield; melting point (mp): 120–121 °C; 400.32 [M^+]; IR: 2939, 2866, 1714, 1654, 1465, 1379, 880, 669 cm^{-1}; UV: inactive; ^{1}H NMR: δ_H 0.71 (s, 3H, H_3-18), 1.12 (s, 3H, H_3-19), 2.42 (m, 2H, H_2-2), 4.07 (d, 1H, *J* = 12 Hz, H-4), 3.83 (d, 1H, *J* = 12 Hz, H-3); ^{13}C NMR: δ_C 212.3, 56.2, 53.7, 46.7, 44.7, 42.5, 39.9, 39.5, 38.5, 38.1, 36.1, 35.7, 35.6, 35.3, 31.7, 28.9, 28.2, 28.0, 24.2, 23.8, 22.8, 22.5, 21.4, 18.6, 12.0; Elemental analysis: $C_{27}H_{44}O_2$ requires C, 80.94%; H, 11.07%; O, 7.99%; found C, 80.96%; H, 11.04%, O, 7.95%.

2.3.2. 4-Chloro-cholest-3-ol-6-one **8**

White solid; 32% yield; melting point (mp): 132–133 °C; 436.65 [M^+]; IR: 3396, 2921, 1713, 1463, 1455, 1377, 1305, 1169, 973, 723, 561 cm^{-1}; UV: inactive; ^{1}H NMR: δ_H 0.71 (s, 3H, H_3-18), 1.12 (s, 3H, H_3-19), 2.68 (dd, 2H, *J* = 3.8 and 15.8 Hz, H_2-2); Elemental analysis: $C_{27}H_{45}O_2Cl$ requires C, 74.19%; H, 10.38%; O, 7.32%; Cl, 8.11%; found C, 74.23%; H, 10.41%, O, 7.29%; Cl, 8.12%.

2.3.3. Treatment of H_2O_2-p-TsOH on Cholest-4(5)en-3,6-diol **6**

The reaction was carried out following the same method as described above by taking 402 mg (1 mmol) of **6** in 50 mL methylene chloride. After purification over a column of silica gel (60–120 mesh, 100 g, 25 cm) it yielded two compounds. One was found identical with cholest-3(4) epoxy-6-one, **7** (eluent 1% EA in PE) and another as the nor lactone, **9** (eluent 2% EA in PE).

2.3.4. A-nor-cholest-6-hydroxy-5,7-olide **9**

White solid; 45% yield; melting point (mp): 144–145 °C; IR: 3442, 2949, 2867, 1767 (γ-lactone), 1735, 1461, 1440, 1375, 1252, 969 cm^{-1}; UV: inactive; ^{1}H NMR: δ_H 0.71 (s, 3H, H_3-18), 1.12 (s, 3H, H_3-19), 4.946 (m, 1H, —OH), 2.32 (q, 2H, *J* = 14.9 Hz, H_2-6), 2.034 (m, 2H, H_2-3). Elemental analysis: $C_{27}H_{44}O_3$ requires C, 77.84%; H, 10.64%; O, 11.52%; found C, 77.80%; H, 10.61%, O, 11.49%.

2.4. Acetylation of cholesterol **1**

Acetylation was carried out following the previous reports [22].

2.5. Oxidation of cholesteryl acetate with sodium dichromate

This reaction was performed following the literature to obtain 7-Keto cholesteryl acetate 10 [22].

2.5.1. 7-Keto cholesteryl acetate 10

White solid (85% yield, eluent 1% EA in PE); IR: 2945, 2868, 1676, 1616, 1460, 1375, 1248, 1228, 867 cm^{-1}; UV: 242 nm; ^{1}H NMR: δ_H 0.68 (s, 3H, H_3-18), 1.21 (s, 3H, H_3-19), 0.88 (d, 3H, *J* = 6.6 Hz, H_3-21), 2.06 (s, 3H, H_3-OAc), 4.21 (m, 1H, H-3), 5.71 (s, 1H, H-6); ^{13}C NMR: δ_C 202, 170.3, 163.8, 126.7, 72.2, 54.7, 49.9, 49.7, 45.4, 43.1, 39.4, 38.6, 38.3, 37.7, 36.1, 35.9, 35.7, 28.5, 28, 27.3, 26.3, 23.8, 22.8, 22.5, 21.2, 21.1, 18.8, 17.2, 11.9.

2.6. Sodium borohydride reduction of 7-Keto cholesteryl acetate **10**

Compound **10** (700 mg, 1.58 mmol) was reduced by sodium borohydride following the method as described above.

2.6.1. 3-Acetoxy cholest-5(6)en-7-ol **11** (30%) and Cholest-5(6)en-3,7-diol **12** (37%)

The analytical data agreed well with the literature report [23,24].

2.7. Treatment of H_2O_2-p-TsOH on 3-acetoxy cholest-4(5)en-7-ol ***11***

Compound **11** (250 mg, 0.56 mmol) was treated with H_2O_2-p-TsOH in methylene chloride following the method as described above.

2.7.1. Cholest-7-keto-3(4),5(6)-diene ***13***

White solid; 20% yield; eluent PE; melting point (mp): 118–119 °C (Lit. 109–119) 382.45 [M^+]; IR: 2945, 2868, 1674, 1616, 1463, 1377, 1331, 1265, 1229, 1191, 1020 cm^{-1}. [25].

2.7.2. Cholest-5(6)epoxy-3,7-diol ***14***

White solid; 25% yield; (eluent 1% EA in PE; melting point (mp): 114–116 °C; 418.56 [M^+]; IR: 3421, 2947, 2869, 1670, 1465, 1379, 1332, 1274, 1170, 1055, 893, 665 cm^{-1}; UV: inactive; ^1H NMR: δ_H 0.68 (s, 3H, H_3-18), 1.21 (s, 3H, H_3-19), 0.88 (d, 3H, J = 6.6 Hz, H_3-21), 3.54 (m, 1H, H-3), 3.87 (m, 1H, H-7), 5.98 (m, 1H, H-6); 2.74 (dd, 2H, J = 9.9 and 15.6 Hz, H_2-2). Elemental analysis: $C_{27}H_{46}O_3$ requires C, 77.46%; H, 11.07%; O, 11.47%; found C, 77.45%; H, 11.11%, O, 11.53%.

2.7.3. Cholest-3(5)epoxy-6,7-diol ***15***

White solid; 32% yield; eluent 2% EA in PE; melting point (mp): 138–139 °C; 418.48 [M^+]; IR: 3423, 2935, 2868, 1718, 1652, 1460, 1380, 1261, 1170, 1053, 887, 792 cm^{-1}; UV: inactive; ^1H NMR: δ_H 0.68 (s, 3H, H_3-18), 1.21 (s, 3H, H_3-19), 0.88 (d, 3H, J = 6.6 Hz, H_3-21), 3.54 (m, 1H, H-6), 3.87 (m, 1H, H-7),4.15 (m, 1H, H-3); 2.74 (dd, 2H, J = 9.9 and 15.6 Hz, H_2-2), Elemental analysis: $C_{27}H_{45}O_3$ requires C, 77.65%; H, 10.86%; O, 11.47%; found C, 77.59%; H, 10.92%, O, 11.53%.

2.8. Treatment of H_2O_2-p-TsOH on Cholest-5(6)en-3,7-diol ***12***

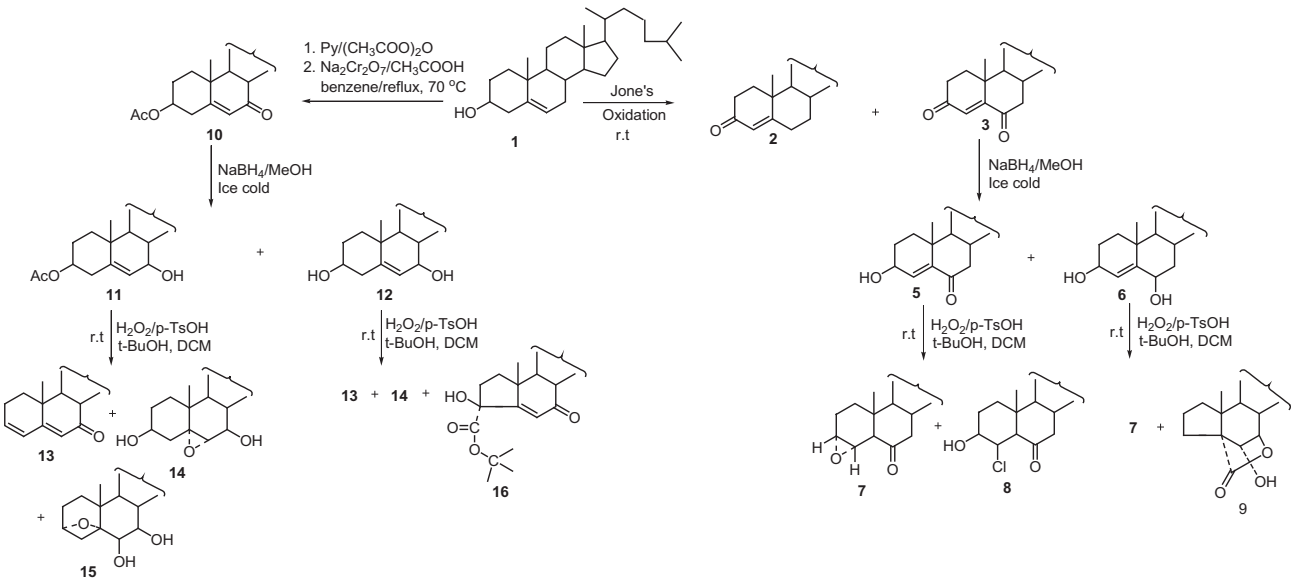
Compound **12** (402 mg, 1 mmol) was treated with H_2O_2-p-TsOH in methylene chloride at room temperature following the method as described above. After purification over a column of silica gel (60–120 mesh, 100 g, 25 cm) it yielded three compounds. First two compounds were found identical with **13** and **14** respectively. The third compound was identified as **16**.

2.8.1. A-nor-3-hydroxy-3-^{t}butoxy-cholest-5(6)en-7-one ***16***

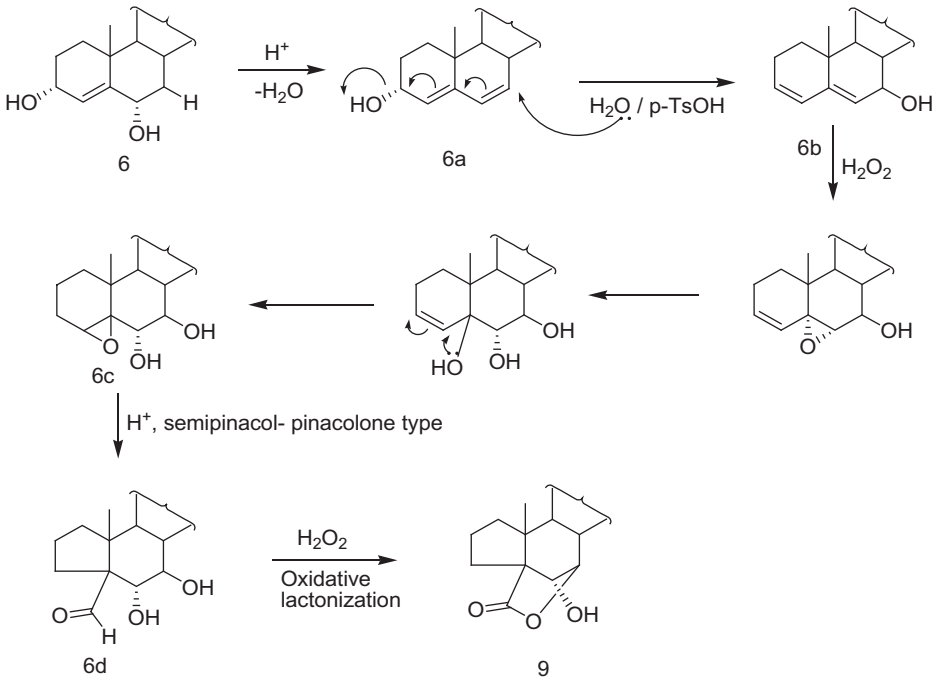
White solid; 47% yield; eluent 3% EA in PE; melting point (mp): 121–123 °C; IR: 3424, 2940, 2867, 1735, 1672, 1461, 1375, 1238, 1148, 1033, 668 cm^{-1}; UV: inactive; ^1H NMR: δ_H 0.68 (s, 3H, H_3-18), 1.21 (s, 3H, H_3-19), 0.88 (d, 3H, J = 6.6 Hz, H_3-21), 2.05 (s, 9H), 4.72 (m, —OH at C_3), 5.22 (s, 1H, H-6); Elemental analysis: $C_{31}H_{50}O_3$ requires C, 79.10%; H, 10.71%; O, 10.20%; found C, 79.15%; H, 10.76%, O, 10.26%.

2.9. Anti-proliferative effect on parasitic protozoa L. donovani and Leishmania major

The cytotoxicity assay was performed on three different species of *Leishmania* in three independent experiments as per the guidelines of biosafety committee of West Bengal State University. *L. donovani* AG83 (MHOM/IN/83/AG83) was originally obtained from Indian Institute of Chemical Biology, Kolkata, India [26]. *L. donovani* LV9 (MHOM/ET/67/HU3/LV9) was obtained from Dr. Bhaskar Saha, NCCS, Pune, India as gift. *L. major* LV39 (MRHO/Sv/59/P strain) was obtained from Dr. Fabienne Tacchini-Cottier, Director, WHO-IRTC, University of Lausanne, Switzerland as gift [27]. The parasites were maintained in animal facility as per the guidelines of institutional animal ethics committee of West Bengal State University. Promastigote morphs of *Leishmania* sp. were transformed from intracellular amastigotes, acquired from splenic aspirates of infected BALB/c mice in complete M 199 medium (Invitrogen) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% FCS (GIBCO) at requisite temperature, 22 °C for *L. donovani* (AG83 and LV9) and at 26 °C for *L. major* LV39. To estimate the percentage of inhibition the derivative, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) micro method was used, as described previously [28]. Briefly, late-log-phase promastigotes were seeded in a 96-well flat-bottom plate (200 µL per well; BD Falcon) in complete M 199 medium in presence or absence of the derivative. To examine the effect of all sixteen compounds (derivative 1 to 16), the cultures were additionally supplemented without (DMSO control) or with increasing concentrations of the derivative (0.5 µg/mL, 1 µg/mL, 2.5 µg/mL and 5 µg/mL) soluble in DMSO (0.2% v/v). Equal volume of DMSO only was added in control experiments. After 72 h of incubation in requisite temperature, MTT (10 mg/mL, 10 µL per well) was added to each well and the plates



Scheme 1. Steroidal allylic rearrangement to different oxysterol derivatives.



Scheme 2. Mechanism for the formation of 9 from 6.

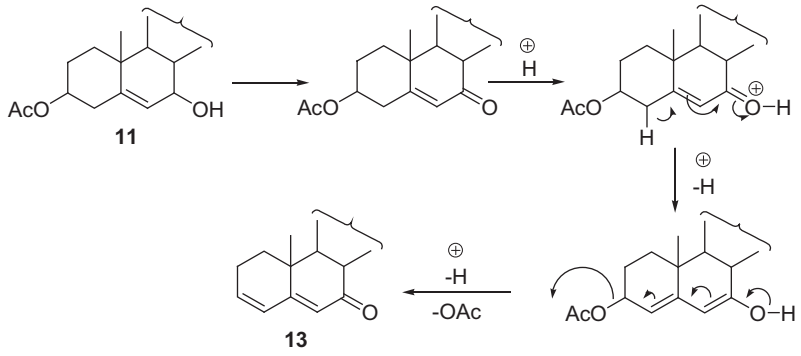
were incubated for an additional 4 h at 37 °C. The enzyme reaction was then stopped by addition of acidic isopropanol (0.4 mL 10(N) HCl in 100 mL isopropanol, 100 µL per well), and the absorbance was measured at 595 nm. Degree of cytotoxicity of conventional antileishmanial drug (reference drug), sodium antimony gluconate (SAG), was also measured on *L. donovani* AG83 [28] at the increasing concentration. Percentage of inhibition was measured in respect to the proliferation of *Leishmania* promastigotes of DMSO control group [28]. Statistical analyses for all experiments were performed by Student's *t* test with the program Sigma Plot using alpha adjustment.

2.10. Analysis of cell cycle progression

The nuclear DNA content of a cell can be quantitatively measured by flow cytometry. Initially, a fluorescent dye that binds stoichiometrically to the DNA is added to a suspension of permeabilized single cells or nuclei. The principle is that the stained material has incorporated an amount of dye proportional to the amount of DNA. The stained material is then measured in the flow cytometer and the emitted fluorescent signal yields an electronic pulse with a height (amplitude) proportional to the total fluorescence emission from the cell. Thereafter, such fluorescence data are considered as a measurement of the cellular DNA content. For flow cytometry analysis of DNA content, exponentially grown *L. donovani* promastigote cells (2×10^6) were incubated without or with IC50 concentrations of compound 3 (1 µg/mL), compound 12 (5 µg/mL) and compound 11 (2.5 µg/mL) in complete M199 media for 48 h. Cells were then harvested and washed three times with 1× PBS, fixed in 45% ethanol (diluted in 1× PBS), treated with 500 µg/mL RNAse A and then suspended in 0.5 M sodium citrate containing 69 µM PI. These samples were analyzed through flow cytometry (FACS Verse, Becton & Dickinson, USA) after keeping them in the dark for 45 min [29].

2.11. DNA fragmentation assay by agarose gel electrophoresis

To determine the fragments of DNA generated during cell death, total cellular DNA was isolated by a previously described [30] and analyzed by agarose gel electrophoresis. Briefly, pellets of 10^7 promastigotes were treated with sarkosyl detergent lysis buffer (50 mM Tris, 10 mM EDTA, 0.5% w/v sodium-*N*-lauryl sarcosine,



Scheme 3. Proposed mechanism for the formation of **13**.

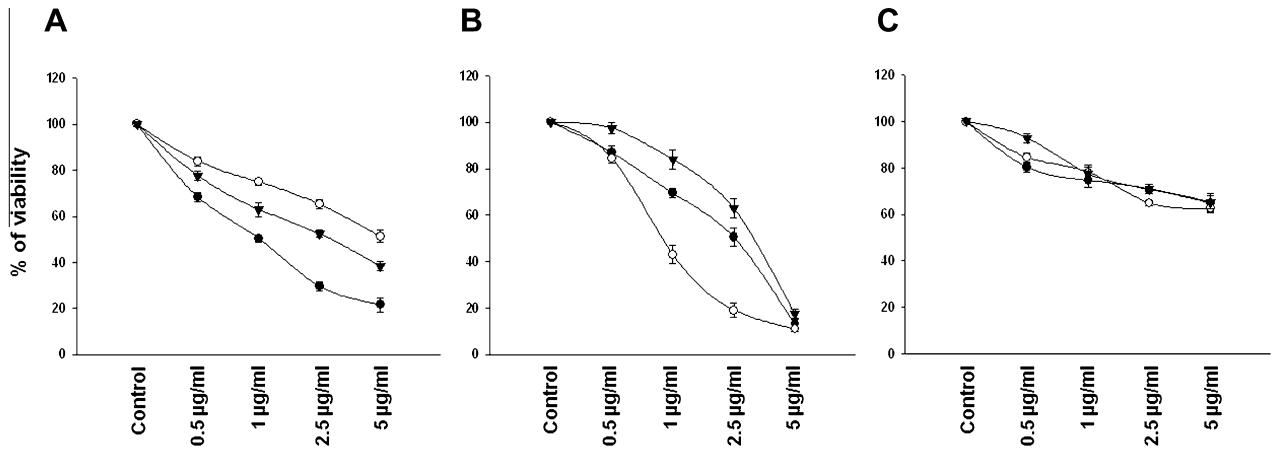


Fig. 1. Determination of 50% inhibitory concentration of active compounds against *Leishmania* promastigotes. *Leishmania* promastigotes were incubated without (control) or with graded concentrations (0.5 μg/mL to 5 μg/mL) of compounds 3 (●), 11 (▼) and 12 (○) in complete M-199 medium for 48 h. Cells were washed and incubated with MTT (10 mg/mL) and percentage of viable *Leishmania donovani* AG83 (A), *Leishmania donovani* LV9 (B) and *Leishmania major* LV39 (C) were estimated as described in materials and methods. Values represent the mean ± SD of three independent experiments in triplicate.

pH 7.5) and proteinase K (15.6 mg mL^{-1}), vortexed and allowed to digest overnight at 50 °C. RNase A (0.3 mg ml^{-1}) treatment was given for 1 h at 37 °C. The lysates were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 16,000*g* for 5 min. The upper phase was treated with 3 M sodium acetate and 100% ethanol for overnight at −20 °C. The sample was centrifuged at 16,000*g* for 10 min, the supernatant removed and 0.5 ml of 70% ethanol added. DNA was solubilized in Tris/EDTA (10 mM/1 mM) buffer and quantitated spectrophotometrically at 260/280 nm. Total DNA was mixed with tracking dye and loaded on 1% agarose gel containing ethidium bromide. Gel was run for 2.5 h at 50 V.

2.12. Determination of anti-proliferative activity against intracellular amastigotes

To check the anti-proliferative effect on amastigotes, macrophages were collected from thioglycolate elicited (1.5 mL of 4% for 5 days) peritoneal exudates of male BALB/c mice as per the guidelines of Institutional Animal Ethics Committee, West Bengal State University. Macrophages were allowed to adhere in 8 chambered slides in complete RPMI 1640 (supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 50 μM 2-ME, 1% essential amino acids and 10% FCS). Macrophages were infected with *L. donovani* AG83 promastigotes (1:10) for 12 h, after washing, further incubated for another 60 h without treatment. After treatment with indicated doses of compound **3**, compound **11** and compound **12** the cultures were terminated after 48 h, fixed with methanol, stained with Giemsa stain and counted under a phase contrast microscope. Extent of infection was expressed as the number of amastigotes per hundred macrophages as described earlier [30].

2.13. Estimation of nitric oxide

Uninfected and *Leishmania*-infected peritoneal macrophages (1×10^6 cells/ml) were treated with indicated doses of compound 3, compound 11 and compound 12 for 48 h. The culture supernatants were collected. The nitrite concentration was measured by Griess reagent (Sigma-Aldrich), as described earlier [18,31].

2.14. Splenocyte proliferation for toxicity assay

Toxicity of compounds towards host cells was assessed on splenocytes *in vitro* using the MTT assay as previously described

Table 1
Anti-proliferative effect of the compounds **3**, **11** and **12** against *Leishmania* promastigotes and reference drug sodium antimony gluconate (SAG).

Compound tested	Concentration (μg/mL)	% of inhibition vs. DMSO control	*P* value
Leishmania donovani AG83 (MHOM/IN/83/AG83)			
Sodium Antimony Gluconate	5	1.375	NS
	10	2.275	NS
	15	5.45	NS
	20	9.8275	NS
	25	10.0275	NS
Leishmania donovani AG83 (MHOM/IN/83/AG83)			
Compound **3**	0.5	31.6	<0.001
	1	49.61	<0.001
	2.5	70.54	<0.001
	5	78.44	<0.001
Compound **12**	0.5	16.06	<0.001
	1	25.07	<0.001
	2.5	34.74	<0.001
	5	48.64	<0.001
Compound **11**	0.5	22.34	<0.001
	1	37.0	<0.001
	2.5	47.57	<0.001
	5	61.67	<0.001
Leishmania donovani LV9 (MHOM/ET/67/HU3/LV9)			
Compound **3**	0.5	13.05	<0.016
	1	30.5	<0.001
	2.5	49.5	<0.001
	5	87.0	<0.001
Compound **12**	0.5	15.5	<0.006
	1	57	<0.001
	2.5	81	<0.001
	5	89.15	<0.001
Compound **11**	0.5	2.5	<0.221
	1	16.0	<0.006
	2.5	37.0	<0.001
	5	82.5	<0.001
Leishmania major LV39 (MRHO/Sv/59/P)			
Compound **3**	0.5	19.76	<0.001
	1	25.31	<0.001
	2.5	29.25	<0.001
	5	35.0	<0.001
Compound **12**	0.5	15.5	<0.006
	1	21.85	<0.001
	2.5	35.15	<0.001
	5	37.5	<0.001
Compound **11**	0.5	7.0	<0.333
	1	22.55	<0.001
	2.5	28.95	<0.001
	5	34.65	<0.001

[32]. Briefly, 1×10^5 splenocytes per well were cultured in 96-well plates in 100 μL complete RPMI 1640 without phenol red and stimulated with phytohemagglutinin (5 μg/mL). Cells were incubated with or without graded concentrations of compound 3, compound 11 and compound 12 (0.5 μg/mL, 1 μg/mL, 2.5 μg/mL and 5 μg/mL). After 48 h of incubation, 20 μL of MTT (5 mg/mL in PBS, Sigma) were added to each well and the cells were incubated for 4 h. The resulting formazan product was dissolved with acid-isopropanol-SDS and the absorbance at a wavelength of 595 nm (A490) was read by iMark Microplate Reader (Biorad, USA). Statistical analyses for all experiments were performed by Student's *t*-test with the program Sigma Plot using alpha adjustment [32].

3. Results and discussion

3.1. Chemistry

In the present study various oxygenated cholesterol derivatives were synthesized by oxidative skeletal rearrangement of steroidal allylic alcohols (Scheme 1) by the Corey's method [33]. Cholesterol does not possess any allylic substitution. To apply Corey's method first allylic substitutions were made in cholesterol skeleton through different approaches to **2**, **3** and **10**. Jone's oxidation of cholesterol gave two conjugated ketones **2** and **3**, **3** being the major product. Compound 3 on reduction ($NaBH_4$) under cold produced a partially reduced product **5** and another fully reduced product, **6** (Vide infra). The structures of these compounds were established by spectral data. Both the synthesized allylic alcohols were then treated with H_2O_2-p-TsOH in DCM at room temperature for 72 h following Corey's method.

Purification of the reaction mixture of **5** yielded two compounds, a 6-ketoepoxide, **7** and the chlorinated one **8**. Such kind of chlorine incorporation by oxidative skeletal rearrangement was reported earlier from our laboratory [34,35]. Compound **6** on the other hand gave **7** and **9**, a γ-lactone as the final rearranged products.

In an attempt, oxidation of cholesteryl acetate with sodium dichromate gave 7-keto cholesteryl acetate, **10** as the final product. Mild borohydride reduction on **10** yields two compounds **11** and

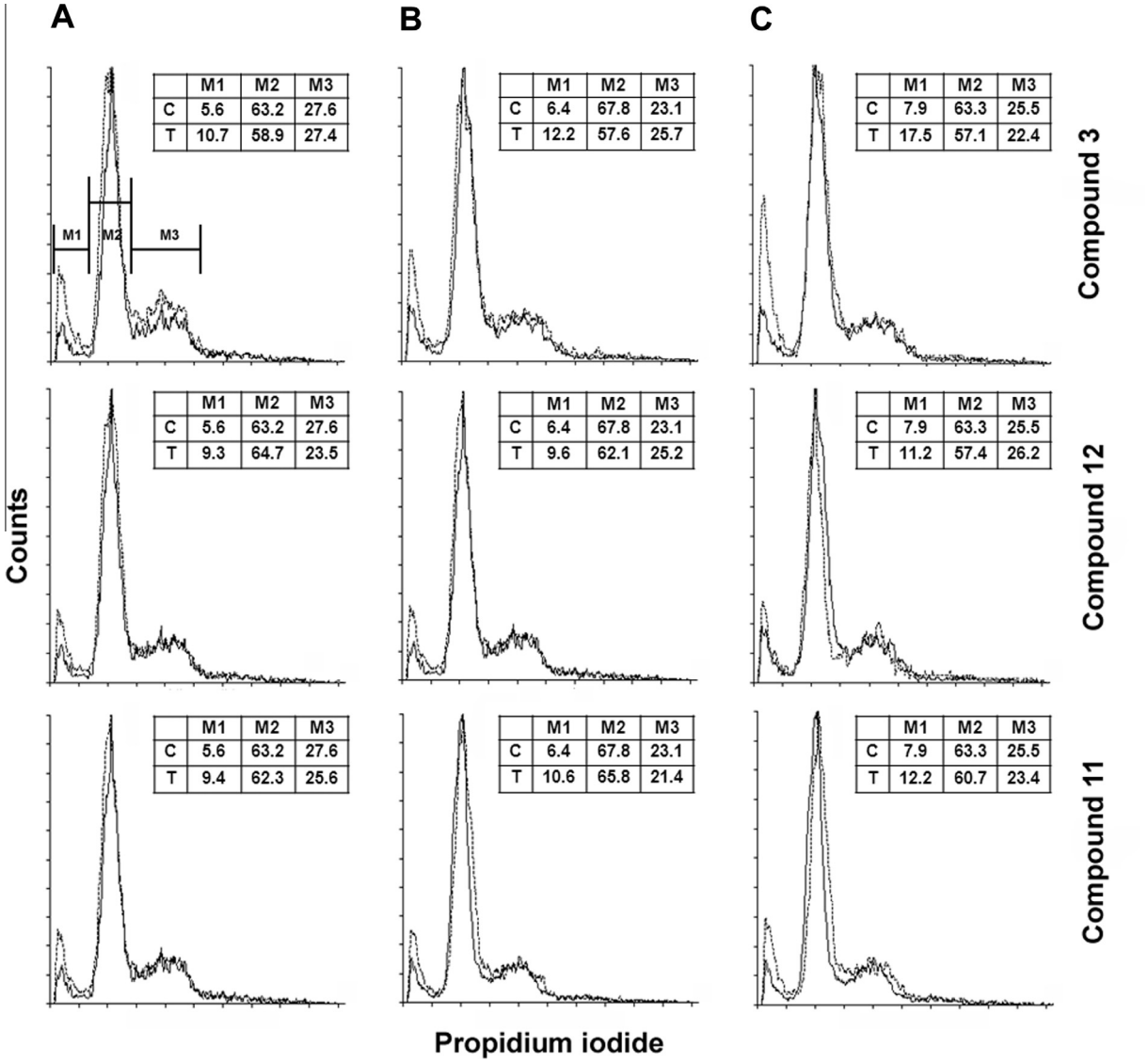


Fig. 2. Compounds 3, 12 and 11 disrupted the cell cycle progression in promastigotes. 2.5×10^6 cells/mL exponential phase *Leishmania donovani* AG83 promastigotes were incubated for 6 h (A), 12 h (B) and 24 h (C) in complete M199 medium in the presence or absence of IC50 concentration of compound **3**, **12** and **11** on promastigotes at 22 °C; washed in 1× PBS and treated with RNase A and PI to analyze the cell cycle progression as described in materials & methods. Values represent the percentage of positive cells (representative of three independent experiments).

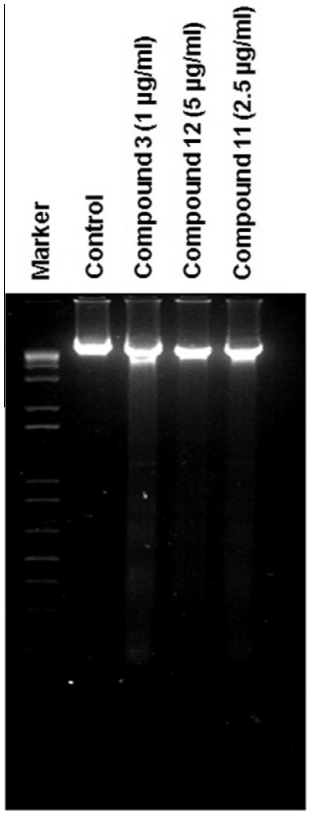


Fig. 3. DNA fragmentation profile in agarose gel from untreated and treated promastigotes with compound **3**, **12** and **11**.

12. Compounds **11** and **12** were then separately treated with H_2O_2-p-TsOH in DCM at r.t for 72 h. Compound **11** yields **13**, **14** and **15** as the rearranged products whereas **13**, **14** and **16** were produced by **12** (Scheme 1). All the new compounds were characterized with the help of spectroscopic analysis (UV, IR, NMR and mass).

3.1.1. Proposed mechanisms

Mechanism proposed here (Scheme 2) is an acid catalyzed carbocationic rearrangement by the involvement of ring A and B of the system. Acid catalyzed dehydration of **6** yielded the intermediate diene **6a** and subsequently **6b**. Reaction of **6b** in presence of H_2O_2 followed by epoxide ring opening gives intermediate diol **6c**. Acid catalyzed rearrangement of **6c** and finally oxidative lactonization of the rearranged product **6d** yielded the lactone **9**. Such kind of rearrangements in case of friedelan skeleton involving C/D rings was also observed [33–35].

Formation of **13** can be explained by the initial oxidation of allylic hydroxyl group to conjugated ketone [34]. The conjugated ketone then undergoes rearrangement via protonation of the carbonyl group followed by elimination of AcOH to form more stable hetero annular dienone 13. Such kind of rearrangement for the steroidal system is already reported in literature [34,35].

Formation of **13** from **12** is also expected in presence of acid through the expulsion of H_2O instead of AcOH as depicted in Scheme 3.

3.2. Anti-leishmanial activity

3.2.1. Anti-proliferative effect on Leishmania promastigotes and estimation of IC50

Anti-proliferative effects of all the oxygenated derivatives were evaluated on *L. donovani*, causative agent of visceral leishmaniasis

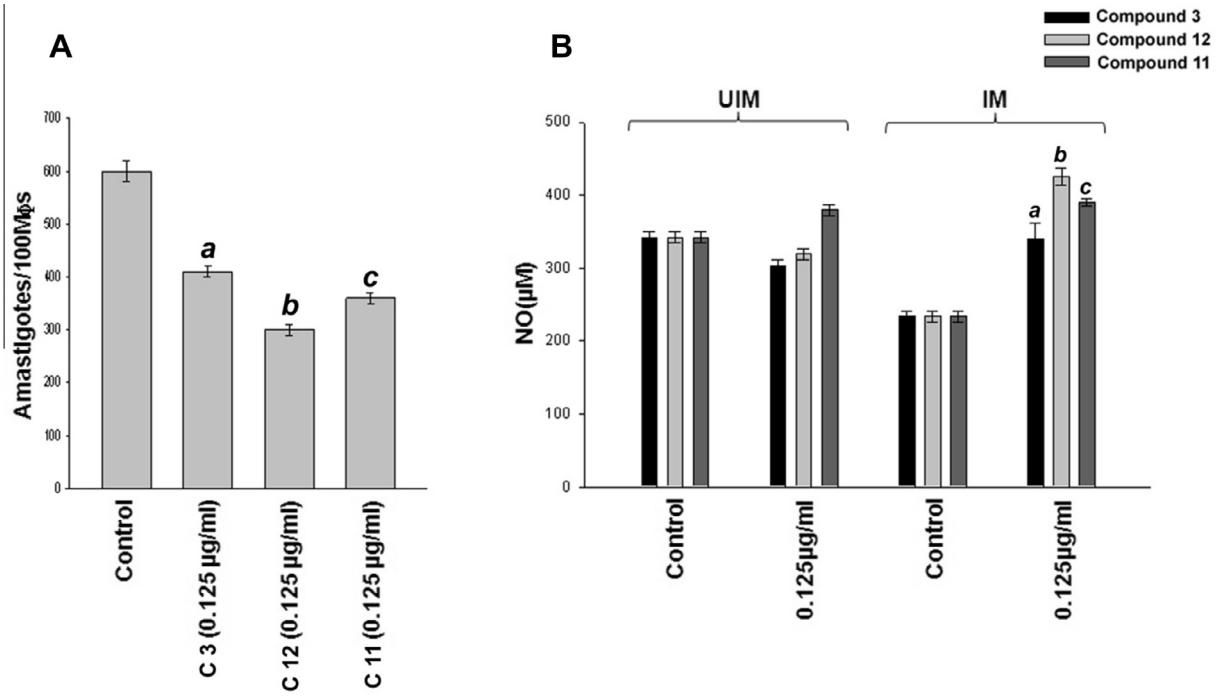


Fig. 4. Anti-survival activities of Compounds **3**, **12** and **11** on intracellular amastigotes and induction of nitric oxide. (A) Resting peritoneal macrophages were infected with *Leishmania donovani* (1:10) and treated without or with compound **3**, compound **12** and compound **11** (0.125 µg/mL). The Giemsa stained cell-micrographs were observed under Carl Zeiss microscope and the anti-leishmanial activity was expressed as the number of amastigotes/100 macrophages. Values represent the mean ± SD of three independent experiments in triplicate [$P^a < 0.01$; $P^b < 0.005$; $P^c < 0.008$]. (B) Nitric oxide was measured by Griess reagent and expressed in µM, as described in materials and methods. Compound **12** was found to induce highest NO at 48 h. Values represent the mean ± SD of three independent experiments in triplicate. $P^a < 0.02$; $P^b < 0.003$; $P^c < 0.002$ vs. DMSO control.

and *L. major*, causative agent of cutaneous leishmaniasis. We found that compounds **3**, **11** and **12** inhibited the proliferation of *L. donovani* AG83, *L. donovani* LV9 and *L. major* LV39 promastigotes *in vitro*. The 50% inhibitory concentration of **3**, **11** and **12** has been estimated against *L. donovani* AG83 promastigotes as 1 μg/mL, 2.5 μg/mL and 5 μg/mL respectively. However, the highest dose (5 μg/mL) of the conventional drug SAG inhibited the promastigote growth only by 1.37% in respect to DMSO control. Significantly, it has been found that **3**, **11** and **12** could also inhibit the proliferation of *L. donovani* LV9 in comparison to DMSO control. However, the effects of the compounds were found costly against *L. major* LV39. Our data clearly indicated that the three compounds have a noteworthy anti-proliferative effect on *L. donovani* AG83 and LV9 strain (Fig. 1 and Table 1).

*3.2.2. Compounds **3**, **11** and **12** disrupted the cell cycle progression in L. donovani promastigotes*

Cell-cycle analysis complemented the cytotoxicity of the compounds obtained from MTT assay. It demonstrated that at 48 h. of culture, IC50 concentration of compounds **3**, **11** and **12** (1 μg/mL, 2.5 μg/mL and 5 μg/mL respectively) caused *L. donovani* promastigotes to remain as resting G0/G1 (M2) cells and inhibited their entry into the S phase (M3). The percentage of dead cells (M1) increased during this incubation period and growth arrest was also visible. At 12 h the test compounds started blockage the entry of *L. donovani* promastigotes in to S phase from G0/G1, however, compound **3** at 24 h completely blocked the entry dose dependently (Fig. 2). Our results suggested that compound **3** preferentially disrupted the cell cycle phases in *L. donovani* promastigotes followed by death *in vitro*.

Tests for another hallmark of apoptosis, the internucleosomal degradation of genomic DNA, showed that the experimental group had nucleosome-sized DNA fragments, giving a DNA-ladder-like pattern identified by agarose gel electrophoresis of DNA from treated cells (Fig. 3).

*3.2.3. Compound **3**, **11** and **12** significantly inhibited the intracellular amastigotes*

Compound **3**, **11** and **12** were evaluated for its ability for inhibiting the intracellular *L. donovani* amastigotes in peritoneal macrophages. Significantly, 0.125 μg/mL compound **3**, compound **11** and compound **12** were found to inhibit the replication of intracellular amastigotes in macrophages by 31.6% ($P < 0.01$), 40% ($P < 0.008$), 50% ($P < 0.005$) respectively (Fig. 4A). Thus compound **12** was found most effective against the *L. donovani* amastigotes, clinically important morphs of this parasite in mammalian host.

*3.2.4. Compound **3**, **11** and **12** significantly induced nitric oxide in macrophages*

Since nitric oxide (NO) is one of the key anti-leishmanial molecules [32], we were interested to check the induction of NO by the three compounds in *Leishmania*-infected and uninfected macrophages (Fig. 4B). Compound **3**, **11** and **12** were found efficient in production of NO in infected-macrophages among which compound **12** was found to induce the highest NO in infected macrophages. The result can be correlated with the highest anti-amastigote competence of this fraction.

*3.2.5. Compound **3**, **11** and **12** were found considerably nontoxic to murine splenocytes*

In order to test the safety of compound **3**, **11** and **12** on mammalian cells, murine splenocytes were treated with these compounds (0.5 μg/mL to 5 μg/mL), and the viability of cells was checked by MTT assay. Compounds were found considerably nontoxic to phytohemagglutinin (5 μg/mL) stimulated BALB/c splenocytes at 96 h (Fig. 5 and Table 2). Least toxic compound **12** towards

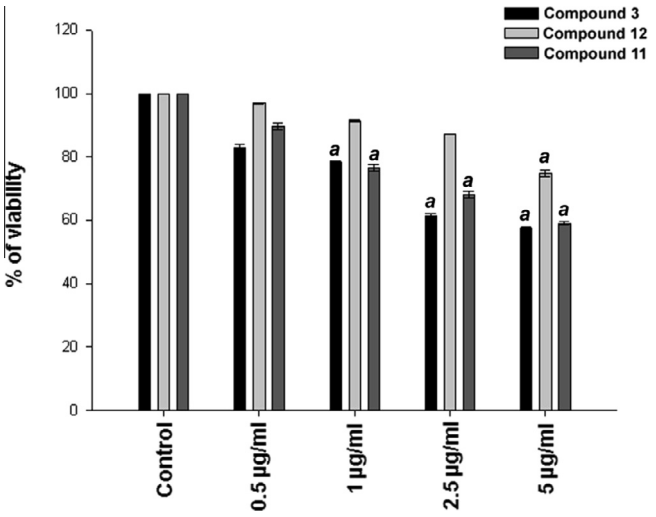


Fig. 5. Splenocyte proliferation assay in presence of compound **3**, compound **12** and compound **11**. MTT survival assay of splenocytes treated by graded concentration of compound **3**, **11** and **12** (0.5–5 μg/mL). Splenocyte proliferation index was determined relative to untreated control cells. Values represent mean ± SD of three independent experiments in triplicate, $P^a < 0.001$ vs. DMSO control.

Table 2
Cytotoxicity assay on murine splenocytes.

Compound tested	Splenocytes	% of inhibition vs. control	*P* value
Compound **3**	0.5	16.93	<0.333
	1	21.56	<0.001
	2.5	38.73	<0.001
	5	42.5	<0.001
Compound **11**	0.5	3.16	<0.333
	1	8.7	<0.333
	2.5	12.83	<0.333
	5	25.33	<0.001
Compound **12**	0.5	10.33	<0.333
	1	23.53	<0.001
	2.5	31.91	<0.001
	5	40.9	<0.001

splenocytes endorsed its best anti-amastigote effect and induction of nitric oxide.

4. Conclusion

In a nutshell, the present paper described the synthesis of different oxysterol derivatives by skeletal rearrangement of steroidal allylic alcohol. Compounds **3**, **11** and **12** showed good activities against different *Leishmania* species. Compound **12** was found least toxic and induced highest NO at 48 h. Least toxicity of compound **12** on splenocytes authorized its best anti-amastigote effect and induction of nitric oxide. The study will definitely help to develop potent, least toxic steroidal anti-leishmanial drug.

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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.2015.12.020.

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