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# Microbial transformation of nandrolone with *Cunninghamella echinulata* and *Cunninghamella blakesleeana* and evaluation of leishmaniacidal activity of transformed products

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ABSTRACT

Therapeutic potential of nandrolone and its derivatives against leishmaniasis has been studied. A number of derivatives of nandrolone (**1**) were synthesized through biotransformation. Microbial transformation of nandrolone (**1**) with *Cunninghamella echinulata* and *Cunninghamella blakesleeana* yielded three new metabolites, 10β,12β,17β-trihydroxy-19-nor-4-androsten-3-one (**2**), 10β,16α,17β-trihydroxy-19-nor-4-androsten-3-one (**3**), and 6β,10β,17β-trihydroxy-19-nor-4-androsten-3-one (**4**), along with four known metabolites, 10β,17β-dihydroxy-19-nor-4-androsten-3-one (**5**), 6β,17β-dihydroxy-19-nor-4-androsten-3-one (**6**) 10β-hydroxy-19-nor-4-androsten-3,17-dione (**7**) and 16β,17β-dihydroxy-19-nor-4-androsten-3-one (**8**). Compounds **1**–**8** were evaluated for their anti-leishmanial activity. Compounds **1** and **8** showed a significant activity *in vitro* against *Leishmania major*. The leishmanicidal potential of compounds **1–8** ($IC_{50}$ = 32.0 ± 0.5, >100, 77.39 ± 5.52, 70.90 ± 1.16, 54.94 ± 1.01, 80.23 ± 3.39, 61.12 ± 1.39 and 29.55 ± 1.14 μM, respectively) can form the basis for the development of effective therapies against the protozoal tropical disease leishmaniasis.

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## 1. Introduction

Biocatalysis is a valuable tool for chemists. A number of stereo-, regio- and chemo-selective transformations have been carried out through biotransformation, which could not be possible through conventional chemical synthesis [1–3]. Various drugs have been subjected to biotransformation in order to obtain novel structural analogues with presumably enhanced biological activities [4,5]. Among biocatalytic agents, the fungi are most extensively used for the biotransformation of various classes of organic compounds of synthetic and natural origin. Stereoslective hydroxylation, epoxidation and oxidation have been reported through the biotransformation with fungi [6–12].

Anabolic steroids are being extensively used for the treatment of various hormonal disorders. We have been using biotransformation techniques to synthesize structurally diverse and pharmaceutically important libraries of steroids. Synthesis of some steroidal derivatives, such as azasterols and amino quinolones, with good leishmanicidal activity, have been reported in the literature. Similarly, infliximab (antibody) has also showed an enhanced leishmanicidal activity when it is used in combination with corticosteroids [13–15].

Nandrolone (19-nortestosterone) (**1**) is an anabolic steroid, known as a performance drug [16–18]. Nandrolone (**1**) has an androgenic activity. It is also used for the treatment of anxiety and memory impairment [19,20]. Nandrolone is commercially marketed as decanoate (deca-durabolin) and phenylpropionate ester (durabolin) derivatives. Deca-durabolin is used for the treatment of osteoporosis and breast cancer.

Leishmaniasis is the second most common vector born tropical disease of protozoal origin. Approximately 350 million people in 88 countries are at the risk [21], with 12 million known cases. The available drugs for the treatment of leishmaniasis, especially for

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oral applications, are too few and often ineffective. The need of development of safe and effective leishmanicidal agents is strongly felt to decrease disease burden and to overcome the side effects and emerging resistance. We have been focusing on the search of new therapeutic agents for leishmaniasis, and synthesizing their structural analogues to enhance their activities [22].

## 2. Experimental

### 2.1. General experimental conditions

Nandrolone (**1**) was purchased from Sigma–Aldrich (cat no. 74640, USA). Sabouraud dextrose agar was procured from Merck KGaA (cat no. 146392, Germany). Thin layer chromatography (TLC) was carried out on precoated plates (Merck KGaA, $PF_{254}$, 20 × 20, 0.25 mm) (Germany). Flash silica was used for column chromatography (CC). Recycling preparative HPLC–LC–908, equipped with JAIGEL–ODS–L–80 ($L$ = 250 mm, I.D. = T20 mm), was used for the purification of compounds. Optical rotations were measured with a JASCO P–2000 polarimeter. UV Spectrum was recorded in chloroform and methanol with an Evolution 300 UV–visible spectrophotometer. Infrared (IR) spectrum was recorded in KBr discs and in chloroform in Vector 22 IR spectrophotometer (Bruker). $^1$H and $^{13}$C NMR spectra were recorded in $CD_3OD$, $CDCl_3$ and DMSO-$d_6$ on Bruker Avance-NMR (300, 500 and 600 MHz). Electron impact mass spectra (EI-MS) were recorded on Jeol JMS-600H mass spectrometer. All the solvents and reagents were of analytical grades.

### 2.2. Microorganisms

The microorganisms were obtained from the American Type Culture Collection (ATCC). *Cunninghamella echinulata* (ATCC 9244) and *Cunninghamella blakesleeana* (ATCC 8688A) were grown on Sabouraud dextrose agar slant and maintained at 4 °C.

### 2.3. Media preparation

Culture medium (1 L each) for *C. echinulata* (ATCC 9244) and *C. blakesleeana* (ATCC 8688A) were prepared by mixing the following ingredients; glucose (10 g), peptone (5 g), $KH_2PO_4$ (5 g), NaCl (5 g) and glycerol (5 mL).

### 2.4. General fermentation and extraction conditions

5 L of the fungal growth media was prepared and distributed equally in 250 mL Erlenmeyer flasks (100 mL each) and autoclaved at 121 °C. The fungal culture was then distributed to 3 seed flasks and incubated for two days on shaker (121 rpm) at 26 ± 2 °C. After suitable growth was observed in the seed flasks, the spores were then transferred to the remaining flasks and placed on shaker (121 rpm, 26 ± 2 °C). Compound **1** (1 g), dissolved in 50 mL of methanol, was distributed among the flasks containing four day old culture of *C. echinulata* (ATCC 9244) and *C. blakesleeana* (ATCC 8688A). The flasks were then placed on shaker for 12 days. Analogous control experiments were conducted which included an incubation of the fungus without substrate **1**, and incubation of **1** in the medium without fungus.

At the end of cultivation phase, the fungal mass was separated by filtration, and washed with dichloromethane (DCM). The filtrate was extracted with DCM (4 L × 3), and after drying with sodium sulfate (anhydrous), concentrated on rotary evaporator to obtain a brown thick material. The control flasks were also processed in the same manner and compared with the test flasks to detect the presence of transformed products.

### 2.5. Fermentation of nandrolone (*1*) with C. echinulata

Nandrolone (**1**) (1 g) was subjected to fermentation with *C. echinulata* for 10 days on a rotatory shaker (121 rpm, 26 ± 2 °C). The solid brown extract (1.5 g), obtained after filtration, extraction and evaporation, was fractionated with silica gel column chromatography. The mobile phase was comprised of 10% gradient of hexanes and ethyl acetate.

Four main fractions (1–4) were obtained from column chromatography. The mobile phase comprises of hexanes: ethyl acetate, and the polarity of mobile phase increases by increasing the ethyl acetate concentration. Fraction 1 was collected over the mobile phase of 25% hexanes and 75% ethyl acetate. Fraction 2 was eluted with 20% hexanes and 80% ethyl acetate. Similarly, fractions 3 and 4 were obtained through column chromatography by using 15% hexanes and 85% ethyl acetate. Fraction 1 was subjected to reverse phase recycling HPLC for purification. The mobile phase was 40% $H_2O$ and 60% MeOH. The compound **5** was eluted at 22 min (2.5 mg) and compound **6** at 31 min (5 mg). Similarly, fraction 2 was injected to normal phase recycling HPLC for the removal of impurities. The mobile phase was 94% chloroform and 6% 2-propanol. The peak over retention time 184 min was collected and dried which yielded 6 mg of compound **4**. Fraction 3 yielded metabolite **2** (2 mg) on elution of silica gel column with hexanes and ethyl acetate (15:85). Fraction 4 yielded metabolite **3** (15 mg, $R_T$ = 204 - min) through purification with reverse phase recycling HPLC using methanol and water as solvent system with 70:30 ratio.

### 2.6. Fermentation of *1* with C. blakesleeana

Incubation, filtration, and extraction procedures were similar as described above. Fermentation of **1** with *C. blakesleeana* yielded four metabolites **4**, **5**, **7** and **8**. Four main fractions (1–4) were acquired after fractionation with silica gel column chromatography. Fraction 1 yielded metabolite **4** (5 mg) on purification with recycling HPLC (chloroform: 2-propanol 94:06 $R_T$ = 184 min), whereas compound **5** was purified from 2 by reverse phase recycling using MeOH and $H_2O$ solvent (60:40, $R_T$ = 42 min). Similarly, metabolites **7** and **8** were purified with silica gel column chromatography. The mobile phase comprises of hexanes and ethyl acetate and the polarity of mobile phase vary by increasing 10% of ethyl acetate in concentration. Compound **7** was eluted at 30% hexanes and 70% ethyl acetate over silica gel column chromatography.

#### 2.6.1. 10β,12β,17β-Trihydroxy-19-nor-4-androsten-3-one (2)

White solid; m. p. 212–214 °C; UV $\lambda_{max}$ 230 nm (MeOH, log $\varepsilon$ 1.97); $[\alpha]_D^{25}$ = −126.2 ($c$ 0.018, MeOH); IR (KBr); $\upsilon_{max}$ 3419 (O–H stretching), 1663 (C=O stretching); HREI-MS $m/z$ 306.1842 ($M^+$, $[C_{18}H_{26}O_4]^+$, calc. 306.1831); EI-MS: $m/z$ 306 [$M^+$] (25), 288 (16), 278 (49), 190 (39); $^1$H NMR ($CD_3OD$, 300 MHz): Table 2; $^{13}$C NMR ($CD_3OD$, 125 MHz): Table 3.

**Table 1**
Leishmanicidal activity of compounds **1**–**8**.

| Compound | Leishmanicidal activity ($IC_{50}$ in μM) |
|---|---|
| **1** | 32.0 ± 0.5 |
| **2** | >100 |
| **3** | 77.39 ± 5.52 |
| **4** | 70.90 ± 1.16 |
| **5** | 54.94 ± 1.01 |
| **6** | 80.23 ± 3.39 |
| **7** | 61.12 ± 1.39 |
| **8** | 29.55 ± 1.14 |
| Standards | |
| Pentamidine | 5.09 ± 0.09 |
| Amphotericin B | 0.29 ± 0.05 |

*2.6.2. 10β,16α,17β-Trihydroxy-19-nor-4-androsten-3-one (3)*

White solid; m. p. 203–205 °C; UV $\lambda_{max}$ 230 nm (MeOH, log $\varepsilon$ 3.4); $[\alpha]_D^{25}$ = 62.5 (*c* 0.01, MeOH); IR ($CHCl_3$); $\upsilon_{max}$ 3388 (O–H stretching), 1664 (C=O stretching); HREI-MS *m/z* 306.1840 ($M^+$, $[C_{18}H_{26}O_4]^+$, calc. 306.1831); EI-MS: *m/z* 306 [$M^+$] (40), 288 (30), 278 (89), 264 (21), 213 (66); $^1$H NMR ($CD_3OD$, 300 MHz): Table 2; $^{13}$C NMR ($CD_3OD$, 150 MHz): Table 3.

*2.6.3. 6β,10β,17β-Trihydroxy-19-nor-4-androsten-3-one (4)*

White solid; m. p. 207–210 °C; UV $\lambda_{max}$ 230 nm (MeOH, log $\varepsilon$ 2.8); $[\alpha]_D^{25}$ = −257.5 (*c* 0.02, MeOH); IR ($CHCl_3$); $\upsilon_{max}$ 3392 (O–H stretching), 1669 (C=O stretching); HREI-MS *m/z* 306.1819 ($M^+$, $[C_{18}H_{26}O_4]^+$, calc. 306.1831); EI-MS: *m/z* 306 [$M^+$] (100), 259 (10), 138 (18.8), 133 (16), 91 (14.4); $^1$H NMR ($CD_3OD$, 300 MHz): Table 2; $^{13}$C NMR ($CD_3OD$, 125 MHz): Table 3.

*2.6.4. 10β,17β-Dihydroxy-19-nor-4-androsten-3-one (5)*

White solid; m. p. 223–225 °C, (230–234 °C, Flines et al. 1963); UV $\lambda_{max}$ 230 nm (MeOH log $\varepsilon$ = 3.2 $[\alpha]_D^{25}$ = −25 (c 0.01, $CHCl_3$), (−28, $CHCl_3$ Flines et al. 1963); IR ($CHCl_3$); $\upsilon_{max}$ 3406 (O–H stretching), 1656 (C=O stretching); HREI-MS *m/z* 290.1900 ($M^+$, $[C_{18}H_{26}O_4]^+$, calc. 290.1882); EI-MS: *m/z* 290 [$M^+$] (60.9), 262 (100.0), 248 (21.2), 148 (69.0), 133 (71.0), 99 (21.7); $^1$H NMR ($CDCl_3$, 300 MHz): Table 2; $^{13}$C NMR ($CDCl_3$, 125 MHz): Table 3.

*2.6.5. 6β,17β-Dihydroxy-19-nor-4-androsten-3-one (6)*

White solid; m. p. 213–215 °C, (209–213 °C, Flines et al. 1963); UV $\lambda_{max}$ 230 nm (MeOH, log $\varepsilon$ 3.34); $[\alpha]_D^{25}$ = −55 (*c* 0.01, EtOH), (−53, EtOH, Flines et al. 1963.); IR ($CHCl_3$); $\upsilon_{max}$ 3389.2 (O–H stretching), 1666 (C=O stretching); HREI-MS *m/z* 290.1897 ($M^+$, $[C_{18}H_{26}O_3]^+$, calc. 290.1882); EI-MS: *m/z* 290 [$M^+$] (23.5), 261 (15.0), 246 (9.3), 213 (27.7), 138 (52.5); $^1$H NMR ($CDCl_3$, 300 MHz): Table 2; $^{13}$C NMR ($CD_3OD$, 150 MHz): Table 3.

*2.6.6. 10β-Hydroxy-19-nor-4-androsten-3,17-dione (7)*

White solid; m. p. 201–204 °C, (195–197 °C, Flines et al. 1963); UV $\lambda_{max}$ 247 nm (MeOH, log $\varepsilon$ 3.38); $[\alpha]_D^{25}$ = 147 (*c* 0.003, $CHCl_3$), (157, $CHCl_3$, Flines et al. 1963); IR ($CHCl_3$); $\upsilon_{max}$ 3413.9 (O–H stretching), 1723 (C=O stretching); HREI-MS *m/z* 288.1725 ($M^+$, $[C_{18}H_{24}O_3]^+$, calc. 288.1725); EI-MS: *m/z* 288 [$M^+$] (7.0), 272 (10.0), 261 (18.8), 138 (53.0); $^1$H NMR ($CDCl_3$, 300 MHz): Table 2; $^{13}$C NMR ($CD_3OD$, 100 MHz: Table 3.

**Table 3**
$^{13}$C NMR Chemical Shift Assignments of ($\delta$ in ppm) compounds **1**–**8**.

| Position | **1**[a] | **2**[b] | **3**[c] | **4**[b] | **5**[d] | **6**[b] | **7**[d] | **8**[d] |
|---|---|---|---|---|---|---|---|---|
| 1 | 27.2 | 34.6 | 32.8 | 34.7 | 33.8 | 27.1 | 33.7 | 26.5 |
| 2 | 37.7 | 33.1 | 34.5 | 34.2 | 33.7 | 37.1 | 33.6 | 36.6 |
| 3 | 202.9 | 202.2 | 202.3 | 202.7 | 199.0 | 203.3 | 200.0 | 199.8 |
| 4 | 124.7 | 124.9 | 124.8 | 126.1 | 124.8 | 125.6 | 124.9 | 124.7 |
| 5 | 170.8 | 168.0 | 168.5 | 162.4 | 164.2 | 168.7 | 163.6 | 167.5 |
| 6 | 36.5 | 31.9 | 33.1 | 73.7 | 31.9 | 72.5 | 31.7 | 35.3 |
| 7 | 31.9 | 34.4 | 34.5 | 39.4 | 31.3 | 39.1 | 31.0 | 30.5 |
| 8 | 41.6 | 35.5 | 35.9 | 30.6 | 35.4 | 34.9 | 34.8 | 39.9 |
| 9 | 51.0 | 53.3 | 54.6 | 54.5 | 52.6 | 50.9 | 52.6 | 49.4 |
| 10 | 43.8 | 70.6 | 70.9 | 72.3 | 70.4 | 39.5 | 70.0 | 42.5 |
| 11 | 27.7 | 29.9 | 20.9 | 20.9 | 20.1 | 27.1 | 19.7 | 25.6 |
| 12 | 37.2 | 79.8 | 38.2 | 37.7 | 36.1 | 37.7 | 30.6 | 36.2 |
| 13 | 44.1 | 49.5 | 43.5 | 44.2 | 43.0 | 44.4 | 47.0 | 44.2 |
| 14 | 51.2 | 49.8 | 47.9 | 51.1 | 50.1 | 50.9 | 50.5 | 47.5 |
| 15 | 24.1 | 24.0 | 36.0 | 24.2 | 23.4 | 24.0 | 21.8 | 33.7 |
| 16 | 30.6 | 30.8 | 70.8 | 30.6 | 30.5 | 30.6 | 35.7 | 78.5 |
| 17 | 82.3 | 82.5 | 81.9 | 82.3 | 81.6 | 82.3 | 220.0 | 89.8 |
| 18 | 11.6 | 6.5 | 12.4 | 11.5 | 10.9 | 11.6 | 13.7 | 12.2 |

[a] = 75 MHz.
[b] = 125 MHz.
[c] = 150 MHz.
[d] = 100 MHz.

*2.6.7. 16β,17β-Dihydroxy-19-nor-4-androsten-3-one (8)*

White solid; m. p. 144–146 °C, (154–156 °C, Flines et al. 1963); UV $\lambda_{max}$ 248 nm (MeOH, log $\varepsilon$ 3.62); $[\alpha]_D^{25}$ = 47 (*c* 0.005, $CHCl_3$), (51, $CHCl_3$, Flines et al. 1963); IR ($CHCl_3$); $\upsilon_{max}$ 3395 (O–H stretching), 1660 (C=O stretching); HREI-MS *m/z* 290.1871 ($M^+$, $[C_{18}H_{26}O_3]^+$, calc. 290.1882); EI-MS: *m/z* 290 [$M^+$] 290 (6), 272 (5), 149 (15), 110 (35), 83 (70). $^1$H NMR ($CDCl_3$, 300 MHz): Table 2; $^{13}$C NMR ($CDCl_3$, 100 MHz): Table 3.

*2.7. Assay for leishmanicidal activity*

Modified NNN biphasic medium, along with normal physiological saline, was used for culturing of *Leishmania major* (DESTO). RPMI 1640 medium was used for culturing of *Leishmania* promastigotes, supplemented with foetal bovine serum (FBS) (10% heat inactivated). Parasites were centrifuged (at log phase) for 10 min at 2000 rpm and washed thrice with saline at same speed and time. Fresh culture medium was used for the dilution of parasites to acquire a final density of $10^6$ cells/mL.

The sample solution was prepared by 1 mg of test compound in 950 μL of RPMI media and 50 μL of DMSO. It was then mixed on

**Table 2**
$^1$H NMR (300 MHz) chemical shift assignments ($\delta$ in ppm, *J* in Hz) of compounds **1**–**8**.

| Position | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** |
|---|---|---|---|---|---|---|---|---|
| 1 | 1.78 m, 1.47 m | 2.19 m, 1.92 m | 1.86 m, 1.03 m | 2.64 m, 2.27 m | 2.12 m, 1.95 m | 2.31 m, 1.87 m | 2.34 m, 1.99 m | 1.54 m, 2.24 m |
| 2 | 1.87 m, 1.10 m | 2.66 m, 2.32 m | 2.58 m, 2.27 m | 1.87 m, 2.16 m | 2.54 m, 2.34 m | 2.37 m, 2.27 m | 2.53 m, 2.17 m | 1.80 m, 1.20 m |
| 3 | – | – | – | – | – | – | – | – |
| 4 | 5.57 m | 5.72 s | 5.72 s | 5.81 s | 5.57 s | 5.85 s | 5.78 s | 5.56 s |
| 5 | – | – | – | – | – | – | – | – |
| 6 | 2.49 m, 2.33 m | 2.21 m, 1.52 m | 2.81 m, 2.32 m | 4.38 m | 2.29 m, 2.58 m | 4.28 t, *J* = 2.64 | 2.65 m, 2.34 m | 2.46 m, 2.24 m |
| 7 | 1.87 m, 1.03 m | 2.60 m, 1.28 m | 2.19 m, 1.87 m | 2.0 m, 1.25 m | 1.84 m, 1.01 m | 1.96 m, 1.23 m | 1.88 m, 1.29 m | 1.77 m, 1.05 m |
| 8 | 1.41 m | 1.80 m | 1.87 m | 1.50 m | 1.75 m | 1.90 m | 1.94 m | 1.35 m |
| 9 | 1.01 m | 1.15 m | 1.07 m | 1.08 m | 1.07 m | 0.87 m | 1.14 m | 0.87 m |
| 10 | 2.21 m | – | – | – | – | – | – | – |
| 11 | 1.53 m, 2.30 m | 1.73 m, 1.64 m | 1.73, 1.67 m | 1.73 m, 1.63 m | 1.64 m, 1.58 m | 1.61 m, 1.34 m | 1.74 m, 1.68 m | 1.84 m, 1.26 m |
| 12 | 2.32 m, 2.25 m | 3.45 dd, *J* = 10.5, 5.7 | 1.87 m, 1.12 m | 1.50 m, 1.20 m | 1.88 m, 1.12 m | 1.85 m, 1.11 m | 1.9 m, 1.12 m | 2.39 m, 2.24 m |
| 13 | – | – | – | – | – | – | – | – |
| 14 | 0.85 td, *J* = 11.6, 4.0 | 0.96 m | 0.85 m | 1.06 m | 0.98 m | 1.01 m | 1.30 m | 1.39 m |
| 15 | 1.62 m, 1.32 m | 1.63 m, 1.40 m | 2.18 m,1,27 m | 1.63 m, 1.36 m | 1.60 m, 1.31 m | 1.61 m, 1.34 m | 1.97 m, 1.58 m | 1.81 m, 1.21 m |
| 16 | 1.98 m, 1.47 m | 2.20 m, 1.52 m | 4.02 q, *J* = 12.48,7.5 | 2.16 m, 2.07 m | 2.05 m, 2.49 m | 2.11 m, 1.25 m | 2.47 m, 2.08 m | 4.04 m |
| 17 | 3.49 t, *J* = 8.8 | 3.81 t, *J* = 8.1 | 3.30 d, *J* = 7.44 | 3.50 t, *J* = 8.4 | 3.6 t, *J* = 8.4 | 3.58 t, *J* = 8.64 | – | 3.5 d, *J* = 5.7 |
| 18 | 0.81 s | 0.84 s | 0.88 s | 0.82 s | 0.79 s | 0.81 s | 0.93 s | 0.81 s |

vortex and dispensed in a 96-well plate. In 96-well microtiter plate, the first row received 180 μL of medium, while remaining wells received 100 μL medium. A 20 μL of the test compound was added in wells containing medium and serially diluted. A 100 μL of parasite culture was transferred in each well. Two rows were used for negative and positive controls. Only growth medium was used for negative controls, while standard leishmanicidal drugs such as amphotericin or pentamidine were used as positive controls. This plate was then incubated at 21–22 °C for 72 h. The culture was examined microscopically on an improved Neubauer counting chamber. Ezfit 5.03 Perella Scientific software (USA) was used for calculation of $IC_{50}$ values of test compounds. All assays were performed in triplicates [23].

## 3. Results and discussion

The biotransformation of nandrolone (**1**), $C_{18}H_{26}O_2$, is being reported for the first time with *C. echinulata* and *C. blakesleeana*. Fermentation with *C. echinulata* afforded five metabolites **2**–**6**, out of which three compounds **2**–**4** were found to be new. Biotransformation of **1** with *C. blakesleeana* generated four compounds **4** and **6**–**8**. Compound **4** is being reported for the first time (Fig. 1).

Metabolite **2** was obtained as a white solid. It was assigned the molecular formula $C_{18}H_{26}O_4$ based on its HREI-MS at *m/z* 306.1842 $[M]^+$ (calc. 306.1831), 32 amu higher than **1**, which indicated dihydroxylation. The IR absorbance showed the presence of hydroxyl (3419 $cm^{-1}$) and carbonyl functionalities (1663 $cm^{-1}$). A downfield methine proton at $\delta$ 3.45 (dd, $J_{a,a}$ = 10.5 Hz, $J_{a,e}$ = 5.7 Hz) appeared in $^1H$ NMR spectrum. The $^{13}C$ NMR spectrum showed two new downfield carbon signals at $\delta$ 70.6 and 79.8, indicating dihyroxylation. The H-4 ($\delta$ 5.72 s), $H_2$-6 ($\delta$ 2.20 m, 1.52 m) and $H_2$-2 ($\delta$ 2.66 m, 2.32 m) showed HMBC correlations with C-10 ($\delta$ 70.6). Through these interactions, the position of one of the two hydroxyl groups was inferred at C-10. Similarly, $H_2$-11 ($\delta$ 1.64, 1.73), H-17 ($\delta$ 3.81) and $H_3$-18 ($\delta$ 0.84) showed HMBC correlations with C-12 ($\delta$ 79.8), indicating the second OH at C-12 (Fig. 2). The $\beta$ (*axial*) stereochemistry of OH at C-10 was assigned on the basis of NOE correlation of H-11 ($\delta$ 1.64 *axial*) with OH ($\delta$ 5.01) at C-10, and NOESY correlation with H-18 ($\delta$ 0.84). The methine proton ($\delta$ 3.45) showed NOESY correlations with H-9, H-14 and H-17, suggesting the $\beta$ hydroxylation at C-12 (*equatorial*) (Fig. 3). The metabolite **2** was identified as 10$\beta$,12$\beta$,17$\beta$-trihydroxy-19-*nor*-4-androsten-3-one.

The HREI-MS of metabolite **3** showed the $[M]^+$ at *m/z* 306.1840 ($C_{18}H_{26}O_4$, calc. 306.1831), indicating dihydroxylation in compound **1**. IR Spectrum showed absorbances at 3352 (O–H) and 1664 $cm^{-1}$ (C=O). The $^1H$ NMR spectrum showed an additional methine proton at $\delta$ 4.02 (q, $J_{e,e}$ = 12.5 Hz, $J_{e,a}$ = 7.5 Hz). The presence of new quaternary ($\delta$ 70.9) and methine ($\delta$ 70.8) carbons in the $^{13}C$ NMR spectrum suggests dihydroxylation in compound **3**. The COSY-DFQF spectrum showed the coupling between the new methine proton ($\delta$ 4.02) and H-17 methine proton ($\delta$ 3.30 d, $J_{a,e}$ = 7.44 Hz). The H-2 ($\delta$ 2.58, 2.27) and H-4 ($\delta$ 5.72) showed HMBC correlations with C-10 ($\delta$ 70.9), which indicated a hydroxylation at C-10. H-15 ($\delta$ 2.18) showed the HMBC correlations with new methine carbon ($\delta$ 70.8) (Fig. 2). This indicated second hydroxylation at C-16. The OH ($\delta$ 4.59) in $^1H$ NMR recorded in DMSO-$d_6$ showed the NOE correlation with H-8 ($\delta$ 1.87), indicating a $\beta$ hydroxylation at C-10 (Fig. 3). The $\beta$ stereochemistry of H-16 was assigned based on NOESY correlation of methine H-16 ($\delta$ 4.02) with $H_3$-18 ($\delta$ 0.88). The structure of compound **3** was deduced as 10$\beta$,16$\alpha$,17$\beta$-trihydroxy-19-*nor*-4-androsten-3-one.

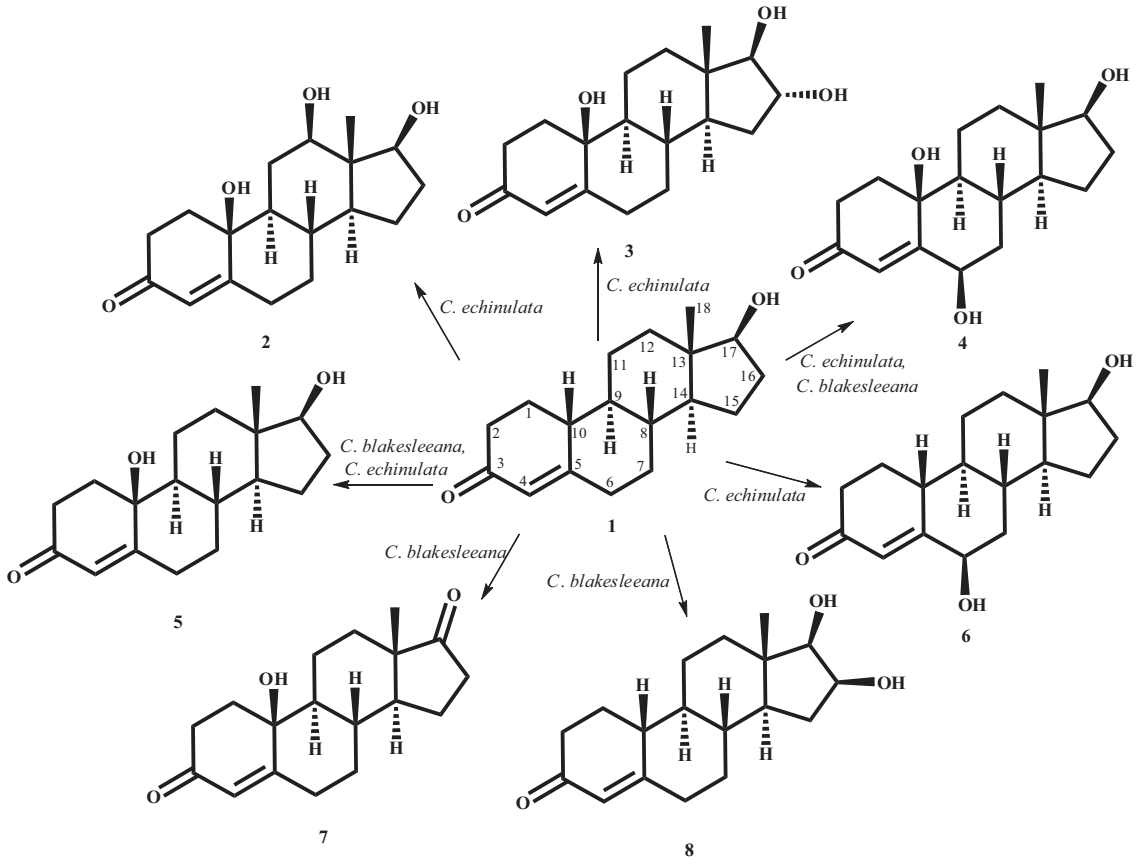


**Fig. 1.** Biotransformation of 1 with *Cunninghamella echinulata* and *Cunninghamella blakesleeana*.

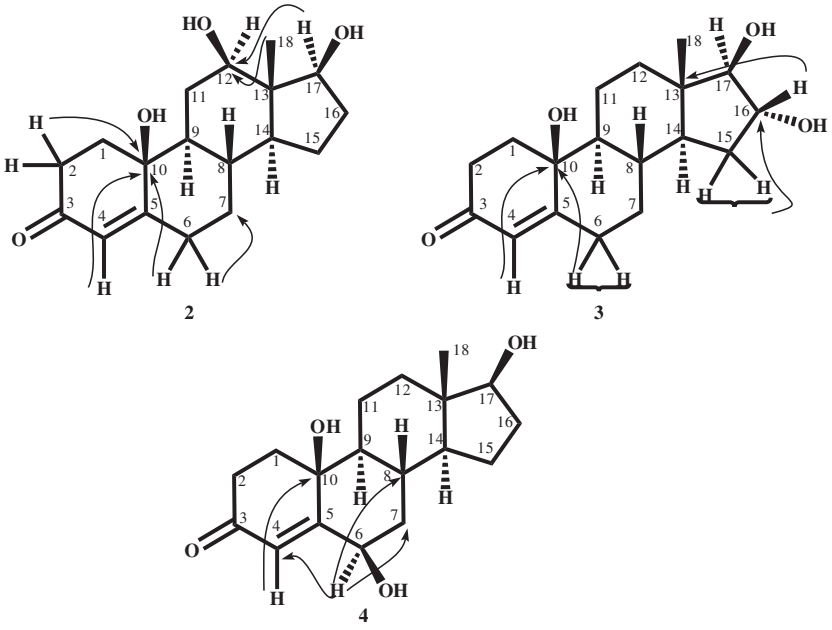

**Fig. 2.** Key HMBC correlations in compounds **2**–**4**.

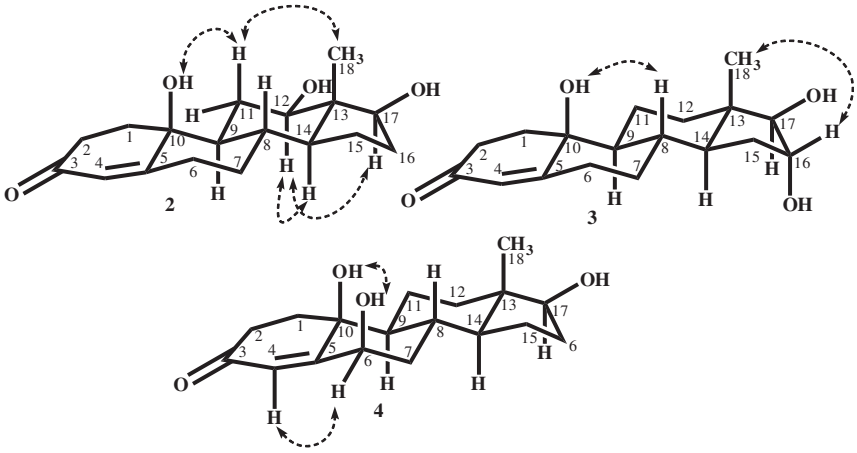

**Fig. 3.** Key NOESY correlations in compounds **2**–**4**.

The HREI-MS of metabolite **4** showed the $[M]^+$ at *m/z* 306.1819 ($C_{18}H_{26}O_4$, calc. 306.1831), which indicated the presence of two additional OH. IR Spectrum showed absorbances at 3392 (hydroxyl group) and 1669 $cm^{-1}$ (ketonic carbonyl group). An additional broad singlet of methine proton was observed at $\delta$ 4.39 ($W_{1/2}$ = 1.2 Hz) in $^1H$ NMR spectrum. The $^{13}C$ NMR spectrum showed two new downfield carbon signals at $\delta$ 73.8 (C-6) and 72.3 (C-10), further indicating dihydroxylation in compound **4**. The H-4 ($\delta$ 5.81) showed HMBC correlations with C-6 ($\delta$ 73.8) and C-10 ($\delta$ 72.3), suggesting OH substituents on C-6 and C-10 (Fig. 2). The methine proton at $\delta$ 4.39 showed HMBC correlations with C-4 ($\delta$ 126.2), C-5 ($\delta$ 162.4) and C-7 ($\delta$ 39.4), indicated an OH at C-6. The C-1 ($\delta$ 2.64, 1.26) and C-2 ($\delta$ 2.18, 1.87) protons showed HMBC correlations with C-10 ($\delta$ 72.3), hence second hydroxylation was inferred at C-10 (Fig. 2). The methine proton at $\delta$ 4.39 showed NOESY correlations with H-4 ($\delta$ 5.81) and $H_2$-7 ($\delta$ 2.01, 1.26). Therefore, $\beta$-OH was placed at C-6. The NOESY correlation of OH ($\delta$ 5.01) (DMSO-$d_6$) with C-6 OH ($\delta$ 4.49) indicated a $\beta$-OH at C-10 (Fig. 3). Thus the metabolite **4** was identified as 6$\beta$,10$\beta$,17$\beta$-trihydroxy-19-*nor*-4-androsten-3-one.

The structures of four known metabolites **5**–**8** were identified by comparison of their reported data with the observed data. The spectral data of compounds **5** and **6** was previously reported by Huszcza et al., Kolet et al., and Flines et al. [24–27], whereas, the data of compounds **7** and **8** was reported by Caspi et al. and Flines et al. [28,29]. The molecular composition of compounds **5, 6** and **8** were 16 amu higher than **1** which showed mono-hydroxylation. Compounds **5, 6** and **8** showed the EI-MS $[M]^+$ *m/z* at 290, whereas compound **7** was at *m/z* 288. Compounds **5–8** were identified as 10$\beta$,17$\beta$-dihydroxy-19-nor-4-androsten-3-one (**5**), 6$\beta$,17$\beta$-dihydroxy-19-nor-4-androsten-3-one (**6**), 10$\beta$-hydroxy-19-nor-4-androsten-3,17-dione (**7**) and 16$\beta$,17$\beta$-dihydroxy-19-nor-4-androsten-3-one (**8**) [24–29]. Compounds **5** and **6** were previously reported by the biotransformation of **1** with *Aspergillus wentii* MRC 200316, whereas compound **7** was synthesized from androstene-3, 17, 19-trione by aromatase cytochrome P-450 mediated transformation. Metabolite **8** was synthesized from **1** on fermentation with molds.

### 3.1. Leishmanicidal activity of nandrolone and its derivatives

We have been focusing on the biotransformation of bioactive steroids and bioactivity evaluation of resulting metabolites. The leishmanicidal activity of steroidal analogues **2–8**, synthesized

through fungal transformation of nandrolone (**1**), is being reported here. These compounds showed a varying degree of leishmanicidal activity, when compared to standard synthetic drugs pentamidine ($IC_{50}$ = 5.09 ± 0.09 μM) and amphotericine B ($IC_{50}$ = 0.29 ± 0.05 μM, Table 1). Compound **8** ($IC_{50}$ = 29.55 ± 1.14 μM) showed a significant leishmanicidal activity (the activity value within $IC_{50}$ range of 5–34 was considered as significant), whereas compound **5** ($IC_{50}$ = 54.94 ± 1.01 μM) was only moderately active (compounds with $IC_{50}$ value between 50 and 60 were considered as moderately active). Compounds **4** ($IC_{50}$ = 70.90 ± 1.16 μM) and **7** ($IC_{50}$ = 61.12 ± 1.39 μM) exhibited a low leishmanicidal activity (compounds showing activity within the $IC_{50}$ range of 60–70 were considered as low active). Some compounds **3** ($IC_{50}$ = 77.39 ± 5.52 - μM) and **6** ($IC_{50}$ = 80.23 ± 3.39 μM) were poorly active (compounds with $IC_{50}$ above 70 were considered as poorly active), whereas compound **2** was inactive (compounds with $IC_{50}$ above 100 were inactive). The metabolite **8** was found to be more active against leishmania, as compared to substrate **1** and other metabolites.

## 4. Conclusion

A number of hydroxylated derivatives **2**–**8** of nandrolone (**1**) were synthesized through microbial fermentation with *C. echinulata* and *C. blakesleeana*. It is an efficient method for the mono- and dihydroxylation of nandolone. The evaluation of nandrolone and its derivatives through an *in vitro* leishmanicidal assay showed a varying range of $IC_{50}$ values. Compounds **1** and **8** were found to be the most active among all compounds with $IC_{50}$ values of 32.0 ± 0.5 and 29.55 ± 1.14 μM, respectively. We are further trying to enhance their leishmanicidal activity by chemical and biological derivatization of **1**.

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