GRAPHICAL ABSTRACT

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4 **Triterpenes from the roots of *Lantana montevidensis* with antiprotozoal activity**
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Abstract

Bioassay guided fractionation of the roots of *Lantana montevidensis* (Verbenaceae) has resulted in the isolation and identification of three new triterpenoids; 13 β -hydroxy-3-oxo-olean-11-en-28-oic acid (**1**), 12 β ,13 β -dihydroxyolean-3-oxo-28-oic acid (**2**) and 12 β ,13 β ,22 β -trihydroxyolean-3-oxo-28-oic acid (**3**) in addition to nine known compounds: oleanonic acid (**4**), oleanolic acid (**5**), 3 β ,25 β -dihydroxy-olean-12-en-28-oic acid (**6**), lantadene A (**7**), 19 α -hydroxy-3-oxo-olean-12-en-28-oic acid (**8**) pomolic acid (**9**), camaric acid (**10**) together with β -sitosterol (**11**) and β -sitosterol-3-*O*- β -D-glucoside (**12**). The structures of the isolated metabolites were elucidated based on comprehensive 1D and 2D NMR spectroscopic data as well as HR-ESI-MS. The extracts and the isolated metabolites were evaluated for their antiprotozoal and antimicrobial activities. Compound **2** showed antibacterial activity against *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* with IC₅₀ values against both organisms of 2.1 μ M and compound **10** showed activity against same organisms with IC₅₀ values 8.74 and 8.09 μ M, respectively, compared to the positive control Ciprofloxacin (IC₅₀=0.3Mm against *Staphylococcus aureus* and MRSA). Compounds **1**, **4**, **5**, **6**, and **10** showed moderate antileishmanial activity with IC₅₀ values ranging between (5.74 - 14.95 μ M) and IC₉₀ values ranging between (11.90 - 19.47 μ M), using Pentamidine as a control (IC₅₀ values 2.09 ->16.8 μ M) and IC₉₀ values ranging between (4.72->16.8 μ M). These compounds also showed highly potent antitrypanosomal activity with IC₅₀ values ranging between (0.39 - 7.12 μ M) and IC₉₀ values ranging between (1.91 – 8.13 μ M), which are more efficient than the DFMO, the antitrypanosomal drug employed as positive control (IC₅₀ and IC₉₀ values 11.82 and 30.82 μ M).

Keywords: *Lantana montevidensis*, Verbenaceae, Triterpenoids, Antiprotozoal activity

1. INTRODUCTION

Trypanosomiasis and leishmaniasis are two debilitating disease groups caused by parasites of *Trypanosoma* and *Leishmania* spp. and affecting millions of people worldwide (**Khan, 2007**). The sandfly serves as the vector for *Leishmania donovani*, which is the etiological agent of visceral leishmaniasis in humans, while the tsetse fly transmits trypanosomes of the *Trypanosoma brucei* which cause sleeping sickness in humans (**TER Kuile BH and Oppendoes, 1992; Barrett and Croft, 2012**). These protozoan parasites account for a considerable burden in terms of public health, mainly but not exclusively in tropical and subtropical countries (**Hammarton et al, 2003**). The morbidity inflicted by these protozoan parasites is the cause of much suffering throughout the tropics and a serious hindrance to socio-economic development in affected countries (**Rodrigues et al, 2014; Hammarton et al, 2003**). Research and development in this area has been essentially abandoned for many decades, and the only drugs on the market often have side effects which would be considered unacceptable nowadays (for example, 5-10% of sleeping sickness patients treated with the arsenical-based drug melarsoprol die not of the disease itself but as a result of the toxicity of the drug treatment) (**Jain and Jain, 2013; Keating**

et al, 2015; Hammarton et al, 2003). Clearly, it is very important to develop novel chemotherapeutic approaches against these diseases.

Genus *Lantana* is represented by 150 species (Munir, 1996). *L. montevidensis* Spreng. (Briq.) is found in many countries as an ornamental plant (Munir, 1996). Infusions of the dried leaves have been used in folk medicine as carminative and antiseptic as well as for the treatment of itching, stomach ache, rheumatism, wounds, toothache, influenza, bronchitis, headaches, and sunstroke (Munir, 1996; Nagão et al, 2002; Wollenweber et al, 1997; Barreto et al, 2010; Montanari et al, 2011). The methanolic extract of the leaves of *L. montevidensis* showed antiproliferative activity against human gastric adenocarcinoma, human uterus carcinoma and murine melanoma cells *in vitro* (Nagão et al, 2002). The ethanolic extracts of the leaves and roots exhibited promising antimicrobial activity (Barreto et al, 2010). Previous studies led to isolation of flavones from the leaves of *L. montevidensis* (Nagão et al, 2002; Wollenweber et al, 1997). Our previous phytochemical studies on *L. montevidensis* included investigation of the volatile oils of the leaves and flowers (^aMakboul et al, 2013), isolation of pentacyclic triterpenoids and flavonoids in addition to the assessment of different leaf extracts and isolated compounds for their biological activities (i.e. Free radical scavenging, antibacterial, anti-inflammatory, analgesic and antipyretic activities) (^bMakboul et al, 2013; Makboul et al, 2014). Within the frame of searching for new bioactive constituents in the roots of *L. montevidensis*, three new pentacyclic triterpenoids (**1-3**), together with nine known compounds (**4-12**) (Figure 1) were obtained. This manuscript describes the isolation and structural elucidation of these new pentacyclic triterpenoids in addition to assessment of the antimicrobial and antiprotozoal activities of the extracts and isolated compounds.

2. RESULTS AND DISCUSSION

Compound **1** was obtained as white amorphous powder. Its negative HR-ESI-MS suggested the molecular formula of C₃₀H₄₆O₄ by the [M-H]⁻ peak at m/z 469.3327 (Calculated for C₃₀H₄₅O₄ 469.3319) and [M-H-H₂O]⁻ peak at m/z 451.3224 (Calculated for C₃₀H₄₃O₃ 451.3213) and more confirmed by positive HR-ESI-MS with [M+H-H₂O]⁺ peak at m/z 453.3371 (Calculated for C₃₀H₄₅O₃ 453.3370). The IR absorption bands at 3492 and 1754 cm⁻¹ indicated the presence of hydroxyl and carbonyl groups, respectively. The ¹H NMR (Table 1) showed signals of seven quaternary methyls resonating at δ_H 0.83, 0.95, 0.98, 1.05, 1.09, 1.17 and 1.24 (each 3H, s), two olefinic protons at δ_H 5.61 (1H, *dd*, 9.6 and 2.0) and 6.09 (1H, *br d*, 9.6). The ¹³C NMR (Table 1) spectrum showed signals equivalent to 30 carbons, appeared in the DEPT experiment as nine quaternary carbons (one oxygen bearing quaternary carbon at δ_C 89.6, one carbonyl group at δ_C 215.7 and a carboxylic group at δ_C 179.6), five methine carbons (two endocyclic olefinic double bond signals at δ_C 128.0 and 135.8), nine methylenes and seven methyl carbons. The provided data strongly recommend compound **1** as an oleanane pentacyclic triterpenoid derivative, though a double bond and a tertiary hydroxyl carbon are unassigned. Placement of the double bond at

$\Delta^{11(12)}$ was emphasized by the HMQC, HMBC and ^1H - ^1H COSY experiments. The HMQC experiments showed a connection between δ_{H} 5.61 (1H, *dd*, 9.6 & 2.0) and δ_{C} 128.0 along with another connection between δ_{H} 6.09 (1H, *br d*, 9.6) and δ_{C} 135.8. The signal at δ_{H} 5.61 was deduced to be H-11 based upon its HMBC correlation (Figure 2) with C-8 (δ_{C} 42.0), C-9 (δ_{C} 53.0) and C-13 (δ_{C} 89.6), while the other proton signal at δ_{H} 6.09 was established as H-12 according to its correlations with C-9 (δ_{C} 53.0), C-13 (δ_{C} 89.6), C-14 (δ_{C} 42.0) and C-18 (δ_{C} 51.0) in the HMBC spectrum. The oxygenated carbon δ_{C} 89.6 was established as C-13 based on presence of correlations between H-11 at δ_{H} 5.61, H-12 at δ_{H} 6.09 and H-27 at δ_{H} 1.09 (3H, *s*) with this carbon. The placement of the ketonic group at C-3 was indicated by the presence of the down field signals at δ_{H} 2.62 (1H, *m*, H-2a) and 2.42 (1H, *m*, H-2b) and the connectivities between the carbonyl δ_{C} 215.7 with Me-23 at δ_{H} 1.17 and Me-24 at δ_{H} 1.05 in HMBC correlations. The relative stereochemistry of the hydroxyl group at C-13 was identified as β -oriented based on the transconfiguration of the C/D ring and further strengthened by NMR data comparison of compound **1** with a similar $\Delta^{11(12)}$ oleanane derivatives (DU et al, 2012; Feng et al, 2012; Huang et al, 1996). Thus, compound **1** was established as 13 β -hydroxy-3-oxo-olean-11-en-28-oic acid which is a new compound. The $\Delta^{11(12)}$ triterpene derivatives are not very common in nature and has only few reports (DU et al, 2012) and this is the first report for them from family Verbenaceae.

Compound **2** was obtained as white amorphous powder with molecular formula $\text{C}_{30}\text{H}_{48}\text{O}_5$ as determined by the positive HR-ESI-MS at m/z 489.3542 $[\text{M}+\text{H}]^+$ (Calculated for $\text{C}_{30}\text{H}_{49}\text{O}_5$ 489.3581) and at 471.3468 $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ (Calculated for $\text{C}_{30}\text{H}_{47}\text{O}_4$ 471.3476). The IR spectrum showed absorption bands at 1740 and 3447 cm^{-1} due to the presence of carbonyl and hydroxyl groups, respectively. Compound **2** was found to be greatly similar to compound **1** except for the absence of the double bond and the presence of additional hydroxyl group. This was shown in the NMR spectrum; ^1H -NMR spectrum (Table 1) revealed the presence of seven tertiary methyls as singlets resonating at δ_{H} 0.90, 1.04, 1.09, 1.19 and 1.31 integrating for 3H each and a singlet resonating at δ_{H} 0.98 integrating for 6H, together with an oxygenated methine at δ_{H} 3.90 (*d*, 6). The ^{13}C -NMR spectrum indicated the presence of seven tertiary methyls, ten methylenes, four methines including an oxygenated methine δ_{C} 76.2 and nine quaternary carbons including a keto group at δ_{C} 217.6 (C-3), a carboxycarbonyl δ_{C} 179.7 (C-28) and an oxygenated quaternary carbon δ_{C} 90.5 (C-13). The HMBC and ^1H - ^1H COSY correlations (Figure 2) strongly recommend the location of the secondary hydroxyl at C-12. This appeared in HMBC spectrum through the correlations between oxygenated methine proton H-12 at δ_{H} 3.90 with δ_{C} 43.8 (C-9), δ_{C} 29.8 (C-11), δ_{C} 90.5 (C-13) and δ_{C} 42.2 (C-14). The ^1H - ^1H COSY experiment showed strong correlations between H-12 at δ_{H} 3.90 together with the adjacent protons: H-11a at δ_{H} 2.10 (*m*) and H-11b at δ_{H} 1.46 (*br d*, 13.6) in addition to presence of correlation between these protons with H-9 at δ_{H} 1.72. The ROESY experiment (Figure 3) was used to determine the configuration of the hydroxyl group at C-12. The presence of cross peaks between H-12 at δ_{H} 3.90 with H-9 at δ_{H} 1.72, CH_3 -27 at δ_{H} 1.31 and CH_3 -29 at δ_{H} 0.98 revealing its α orientation. This assignment was

furthermore confirmed by the absence of any connectivities with H-18 at δ_H 2.02 or CH_3 -26 at δ_H 1.19 which are known to be in β orientation. By comparison of the NMR data of compound **2** with that of 12 α ,13-dihydroxyolean-3-oxo-28-oic acid (Lui et al, 2005; Tao and Fang, 2012) and 12 hydroxy β oriented oleanane derivatives (Irungu et al, 2014), this compound was established as 12 β ,13 β -dihydroxyolean-3-oxo-28-oic acid which is a newly reported triterpenoid.

Compound **3** was obtained as white amorphous powder with molecular formula $\text{C}_{30}\text{H}_{48}\text{O}_6$ as determined by the HR-ESI-MS at m/z 505.3537 $[\text{M}+\text{H}]^+$ (Calculated for $\text{C}_{30}\text{H}_{49}\text{O}_6$ 505.3530) and at m/z 487.3468 $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ (Calculated for $\text{C}_{30}\text{H}_{47}\text{O}_5$ 487.3425) and NMR analysis. Compound **3** was found to have great similarity with compound **2** except for presence of additional hydroxyl group. The ^1H and ^{13}C - NMR spectra (Table 1) showed signals referring the presence of seven methyls, nine methylenes, five methines that two of them are oxygenated; δ_H 3.90 (1H, *d*, 6) / δ_C 76.4 (C-12) and δ_H 4.10 (1H, *br s*) / δ_C 75.8 (C-22) and nine quaternary carbons including a keto group at δ_C 217.7 (C-3), a carboxycarbonyl δ_C 179.9 (C-28) and an oxygenated-quaternary carbon δ_C 90.6 (C-13). The ^1H - ^1H COSY spectrum and HMBC experiment suggest (Figure 2) the placement of the additional hydroxyl group in C-22. The HMBC experiment showed long-range correlations between the methyl protons at C-20 (δ_H 0.89 and 0.97) and C-21 (δ_C 39.8), and also the ^1H - ^1H COSY spectrum showed a correlation between H-22 α at δ_H 4.10 with H-21- α at δ_H 1.55 (1H, *m*) and H-21- β at δ_H 1.42 (1H, *m*), indicating the location of the hydroxyl group at C-22. In addition to the presence of correlations between H-22 α at δ_H 4.10 together with δ_C 28.2 (C-16), δ_C 51.3 (C-17) and δ_C 58.2 (C-18) in HMBC spectrum. This was also further confirmed by the downfield shift of H-18 to δ_H 3.00 which was reported to be characteristic for the C-22 oxygenated Oleanane derivatives (^bMakboul et al, 2013; Begum et al, 2008; Siddiqui et al, 1995). Furthermore, its β -orientation was confirmed by the correlations in the ROESY spectrum (Figure 3) of H-22 at δ_H 4.10 with H-12 (δ_H 3.90), CH_3 -27 (δ_H 1.30) and CH_3 -29 (δ_H 0.97). By comparison of the NMR spectral data of compound **3** with similar compounds having oxygenation at positions 12, 13 and/or 22 in literature (^bMakboul et al, 2013; Lui et al, 2005; Tao and Fang, 2012; Siddiqui et al, 1995), this compound was established as 12 β ,13 β ,22 β -trihydroxyolean-3-oxo-28-oic acid. Reviewing the current literature, compound **3** was found to be a new triterpenoid.

Compounds **4-12** were identified as oleanonic acid (**4**) (Huang and Huang, 2004), oleanolic acid (**5**) (Juang et al, 2005), 3 β ,25 β -dihydroxy-olean-12-en-28-oic acid (**6**) (He et al, 2011), lantadene A (**7**) (Juang et al, 2005), 19 α -hydroxy-3-oxo-olean-12-en-28-oic acid (**8**) (Fraga et al, 2006), pomolic acid (**9**) (Huang and Huang, 2004), camaric acid (**10**) (Siddiqui et al, 1995) together with β -sitosterol (**11**) (Kamboj and Saluja, 2011) and β -sitosterol-3-*O*- β -D-glucoside (**12**) (Rahmana et al, 2009), respectively, by comparison of their ^1H and ^{13}C -NMR spectroscopic data with those reported in the literature. Compounds **4-10** were isolated from *L. montevidensis* for the first time. It is worthy to mention that this is the second time for the isolation of compound **8** from nature and the first time to be reported from family Verbenaceae.

All of the compounds were tested for antimicrobial activity except compound **3** due to lack of the amount. Only compounds **2** and **10** were found to have a high antibacterial activity. Compound **10**, showed potent activity against *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) with IC₅₀ values of 8.74 and 8.09 µM, respectively, while compound **2**, showed even better activity against same organisms with IC₅₀ values of 2.1 µM for both organisms, [Ciprofloxacin showed IC₅₀=0.3 µM against *Staphylococcus aureus* and MRSA].

The antiprotozoal activity of compounds **1**, **4**, **5**, **6** and **10** were evaluated *in vitro* against *L. donovani* promastigotes, axenic amastigotes and intracellular amastigotes in THP1 cells. The compounds were also evaluated against *T. brucei brucei* trypomastigote forms. All the compounds were simultaneously tested against THP1 cell for determination of general cytotoxicity. Compound **4** showed moderate antileishmanial activity against the promastigotes with IC₅₀ value of 11.44 µM. Compounds **4** and **10** were significantly active against leishmania amastigotes with IC₅₀ values of 13.90 and 2.54 µM and IC₉₀ values of 19.47 and 15.15 µM, respectively. Compounds **4**, **6** and **10** were found to be active against intracellular leishmania amastigotes in THP1 cells with IC₅₀ values of 5.74, 14.95 and 10.89 µM, respectively. Regarding antitrypanosomal activity, compounds **1**, **4**, **5**, **6** and **10** showed potent activity toward *T. brucei brucei* with IC₅₀ values of 5.14, 0.39, 5.67, 7.12 and 3.20 µM, respectively, and with IC₉₀ values of 6.66, 1.91, 7.20, 10.51 and 8.13 µM, respectively. Compounds **4** and **10** showed mild cytotoxicity against THP1 cells with IC₅₀ values of 16.01 and 12.57 µM, respectively. Pentamidine was used as a positive control drug for antileishmanial assays (Table 2). DMFO was used as a positive control drug for antitrypanosomal assay, which showed IC₅₀ and IC₉₀ values of 11.82 and 30.82 µM. The aqueous and chloroform extracts of *L. montevidensis* showed mild antileishmanial activity against *L. donovani* axenic amastigotes only and potent antitrypanosomal activity against *T. brucei* trypomastigotes culture (Table 3).

3. EXPERIMENTAL SECTION

3.1. General Experimental.

Optical rotations were obtained using a Research Analytical Autopol V polarimeter (Rudolf), UV spectra were recorded on Perkin–Elmer 3B UV/Visible spectrophotometer and IR spectra were measured on a tensor 27, Bruker. All NMR experiments were carried out on a Varian - Mercury AS 400 MHz NMR spectrometer. High resolution mass spectra were obtained by a Bruker BioApex spectrometer USA. Column chromatography was carried on Silica gel G₆₀ (60-120 mesh, Merck), Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan). Solid phase extraction (SPE) cartridge (Supelco reversed phase C-18, 10g). Analytical TLC was conducted on precoated aluminum sheets silica 60 F₂₅₄, 0.25 mm and reversed phase RP-18 F₂₅₄ (E-Merck, Darmstadt, Germany).

3.2.Plant material.

The roots of *Lantana montevidensis* (Spreng.) Briq. were collected during the flowering stage in the period of February–November 2013 from the gardens of Assiut University, Assiut, Egypt. A voucher sample (No. 2009 LM) has been deposited in the Herbarium of Pharmacognosy Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt.

3.3.Extraction and isolation of constituents.

The air-dried powdered roots of *L. montevidensis* (1.5 Kg) were exhaustively extracted using 70% MeOH (5L x 4) by maceration at room temperature for five days. The methanolic extract was concentrated under reduced pressure to afford a dark brown viscous residue (112 g) which was suspended in distilled water (200 ml) and partitioned using CHCl₃ (1L x 4) to yield two major fractions, the chloroformic fraction (40 g) and aqueous fraction (70 g). The chloroformic fraction was subjected to VLC on silica gel (750 g) using mixtures of Hexane- EtOAc as eluent in the order of increasing polarities to yield six fractions (I–VI). Fraction I (3 g, eluted with *n*-hexane-EtOAc, 9.5:0.5), II (5.4 g, with *n*-hexane-EtOAc, 9:1), III (10 g, with *n*-hexane-EtOAc, 8:2), IV (10.8 g with *n*-hexane-EtOAc, 7:3), V (4.6 g, with *n*-hexane-EtOAc, 6:4) and VI (3.5 g with *n*-hexane-EtOAc, 5:5). Fraction I was rechromatographed over silica gel (90 g) CC [3 (ID) x 100 (L) cm] using *n*-hexane-EtOAc in gradient elution to afford compounds: **11** (100 mg) and **7** (7 mg). Fraction II was subjected to silica gel (150 g) CC [5 (ID) x 150 (L) cm] using *n*-hexane-EtOAc in gradient elution to afford two subfractions II-1 (2.3 g) and II-2 (1.7 g). Repeated crystallization of each of the two subfractions from acetone gave compound **1** (2.5 mg) and compound **8** (5 mg), respectively. Fraction III was purified using silica gel (300 g) CC [5 (ID) x 180 (L) cm] using *n*-hexane-EtOAc mixtures as mobile phase resulting in three subfractions III-1 (2 g), III-2 (6.3 g) and III-3 (400 mg). Repeated crystallization of subfraction III-1 from acetone yielded compound **4** (1.5 g), while repeated crystallization of subfraction III-2 from acetone gave compound **5** (6 g). Subfraction III-3 was rechromatographed on Solid phase extraction (Reversed phase C-18, 10 g) using MeOH-H₂O as mobile phase; the fractions eluted with MeOH-H₂O (70:30) were collected together and concentrated to yield compound **9** (10 mg). About 5 g of fraction IV was subjected to silica gel (180 g) CC [5(ID) x 170 (L) cm] using *n*-hexane- EtOAc mixtures as eluent. Fractions eluted with *n*-hexane-EtOAc (7.5:2.5) were collected together and concentrated to yield subfraction IV-1 (300 mg). Subfraction IV-1 was rechromatographed on Sephadex LH-20 (30 g) CC [3 (ID) x 80 (L) cm] using MeOH as eluent to yield two compounds: **10** (13 mg) and **2** (3 mg). Fraction V was purified using silica gel (180 g) CC [5 (ID) x 170 (L) cm] using CHCl₃-EtOAc mixtures as mobile phase resulting in two subfractions V-1 (1g, eluted with CHCl₃-EtOAc, 8:2) and V-2 (2.4g, eluted with CHCl₃-EtOAc, 1:1). Subfraction V-1 was rechromatographed on silica gel (30g) CC [3 (ID) x 70 (L) cm] using CHCl₃-MeOH mixtures as mobile phase. The fractions eluted with CHCl₃-MeOH (9.5:0.5) were collected and concentrated to yield compound **6** (15 mg). Subfraction V-2 was subjected to silica gel (75 g) CC [3 (ID) x 90 (L) cm] using CHCl₃-EtOAc mixtures as eluent. The fractions eluted

with CHCl₃ and CHCl₃-EtOAc (8:2) were collected and concentrated to yield compounds **3** (1.9 mg) and **12** (50 mg).

3.4. Spectroscopic data of compounds 1-3:

3.4.1. 13 β -hydroxy-3-oxo-olean-11-en-28-oic acid: Amorphous powder, $[\alpha]_D^{20} + 43.991$ (c 0.05, MeOH); IR (NaCl / Film) $\nu_{\max}$ 1460, 1708, 1754, 2921 and 3492 cm⁻¹. UV(MeOH) $\lambda_{\max}$ 206 nm¹H-NMR (C₅D₅N, 400 MHz) and ¹³C-NMR (C₅D₅N, 100 MHz) data, **see Table 1**; negative HR-ESI-MS m/z 469.3327 [M-H]⁻ (Calculated 469.3319) and m/z 451.3224 [M-H-H₂O]⁻ (Calculated 451.3213) and m/z 453.3371 [M+H-H₂O]⁺ (Calculated 453.3370).

3.4.2. 12 β ,13 β -dihydroxyolean-3-oxo-28-oic acid: amorphous powder; $[\alpha]_D^{20} + 41.891$ (c 0.05, MeOH); IR(NaCl / Film) $\nu_{\max}$ 1646, 1741, 2924 and 3487 cm⁻¹; UV(MeOH) $\lambda_{\max}$ 201 nm; ¹H (CDCl₃, 400MHz) and ¹³C NMR (CDCl₃, 100 MHz) data, **see Table 1**; positive HR-ESI-MS at m/z 489.3542 [M+H]⁺ (Calculated 489.3581) and at m/z 471.3468 [M+H-H₂O]⁺ (Calculated 471.3476).

3.4.3. 12 β ,13 β ,22 β -trihydroxyolean-3-oxo-28-oic acid: amorphous powder; $[\alpha]_D^{20} + 41.776$ (c 0.05, MeOH); IR(NaCl / Film) $\nu_{\max}$ 1642, 1740, 2925, 3447 cm⁻¹; UV(MeOH) $\lambda_{\max}$ 203nm; ¹H (CDCl₃, 400MHz) and ¹³C NMR (CDCl₃, 100 MHz) data, **see Table 1**; positive HR-ESI-MS at m/z 505.3537 [M+H]⁺ (Calculated 505.3530) and at m/z 487.3468 [M+H-H₂O]⁺ (Calculated 487.3425).

3.5. Antimicrobial Bioassays.

Pure compounds were tested for antimicrobial activity against the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, and *Aspergillus fumigates* ATCC 204305, as well as the bacteria methicillin-resistant *Staphylococcus aureus* ATCC 33591, *Cryptococcus neoformans* ATCC 90113, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068 (Bharate et al, 2007; Radwan et al, 2007; Ma et al, 2004). Amphotericin B (ICN Biomedicals, Aurora, Ohio) for fungal and ciprofloxacin (ICN Biomedicals) for bacterial bioassays were used as positive controls, respectively.

3.6. Antiprotozoal Assay.

The *L. montevicensis* extracts and isolated compounds were tested for their antiprotozoal activities against *L. donovani* promastigotes, axenic amastigotes and intracellular amastigotes in THP1 cells. The compounds were also evaluated against *T. brucei brucei* trypomastigote forms. All the compounds were simultaneously tested against THP1 cell for determination of general cytotoxicity employing the methods described previously (Hamid et al, 2004; Manda et al, 2014). The *in vitro* antileishmanial and antitrypanosomal assays were done on cell cultures of *L.*

donovani promastigotes, axenic amastigotes, THP1-amastigotes and *Trypanosoma brucei* trypomastigotes by Alamar Blue assays as described earlier. The assays have been adapted to 384 well micro-plate, the samples with appropriate dilution were added to the *L. donovani* promastigotes or *L. donovani* amastigotes or *T. brucei* trypomastigotes cultures (2×10^6 cell/mL). The compounds were tested at three concentrations ranging from 40-1.6 $\mu\text{g/mL}$. The plates were incubated at 26 °C for 72 h (37 °C for axenic amastigotes and *T. brucei* trypomastigotes) and growth of the parasites in the cultures was determined by Alamar Blue assay (Manda et al, 2014). Also, the compounds were also tested against *L. donovani* intracellular amastigotes in THP1 cells employing a parasite rescue and transformation assay. The compounds were simultaneously tested for cytotoxicity against THP1 cell cultures. The conditions for seeding the THP1 cells, exposure to the test compounds and evaluation of cytotoxicity were the same as described in parasite-rescue and transformation assay (Jain et al, 2012). IC₅₀ and IC₉₀ values were computed from the dose response curves using XLfit software. Pentamidine and DMFO were used as positive controls.

4. ACKNOWLEDGMENTS

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5. SUPPORTING INFORMATION

NMR and HR-ESI-MS spectrum are available for compounds 1-12.

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Table (1): ¹H and ¹³C-NMR spectral data of compounds 1, 2 and 3^a:

Position	1		2		3	
	δ_H (m, J in Hz)	δ_C type	δ_H (m, J in Hz)	δ_C type	δ_H (m, J in Hz)	δ_C type
1	1.98, <i>m</i> 1.34, <i>m</i>	39.0 <i>t</i>	1.66, <i>m</i> 1.47, <i>m</i>	39.5 <i>t</i>	1.64, <i>m</i> 1.46, <i>m</i>	39.8 <i>t</i>
2	2.62, <i>m</i> 2.42, <i>m</i>	34.7 <i>t</i>	2.56, <i>ddd</i> (16.5, 7.6 2.8) 2.45, <i>m</i>	34.0 <i>t</i>	2.57, <i>brd</i> (16.3, 7.3) 2.44, <i>m</i>	34.1 <i>t</i>
3	--	215.7 <i>s</i>	--	217.6 <i>s</i>	--	217.7 <i>s</i>
4	--	47.7 <i>s</i>	--	47.3 <i>s</i>	--	47.5 <i>s</i>
5	1.36 <i>d</i> (3.6)	54.5 <i>d</i>	1.37, <i>d</i> (4)	54.8 <i>d</i>	1.36, <i>d</i> (4.2)	55.0 <i>d</i>
6	1.56, <i>br d</i> (2.8) 1.41, <i>m</i>	19.2 <i>t</i>	1.57, <i>m</i> 1.36, <i>m</i>	19.1 <i>t</i>	1.56, <i>m</i> 1.37, <i>m</i>	19.2 <i>t</i>
7	1.32, <i>m</i> 1.38, <i>m</i>	31.0 <i>t</i>	1.62, <i>m</i> 1.25, <i>m</i>	33.3 <i>t</i>	1.61, <i>m</i> 1.24, <i>m</i>	33.5 <i>t</i>
8	--	42.0 <i>s</i>	--	42.2 <i>s</i>	--	42.3 <i>s</i>
9	1.99, <i>brs</i>	53.0 <i>d</i>	1.72, <i>brd</i> (12.6)	43.8 <i>d</i>	1.72, <i>brd</i> (12.4)	44.0 <i>d</i>
10	--	36.2 <i>s</i>	--	36.2 <i>s</i>	--	36.3 <i>s</i>
11	5.61 <i>dd</i> (9.6 and 2.0)	135.8 <i>d</i>	2.10, <i>m</i> 1.46, <i>brd</i> (13.6)	29.7 <i>t</i>	2.09, <i>m</i> 1.46, <i>brd</i> (13.4)	29.9 <i>t</i>
12	6.09 <i>brd</i> (9.6)	128.0 <i>d</i>	3.90, <i>d</i> (6)	76.2 <i>d</i>	3.90, <i>d</i> (6)	76.4 <i>d</i>
13	--	89.6 <i>s</i>	--	90.5 <i>s</i>	--	90.6 <i>s</i>
14	--	42.0 <i>s</i>	--	42.2 <i>s</i>	--	42.3 <i>s</i>
15	1.14, <i>m</i> 1.78, <i>m</i>	28.0 <i>t</i>	1.64, <i>dd</i> (9.2, 4) 1.40, <i>m</i>	27.4 <i>t</i>	1.65 <i>dd</i> (9.2, 4) 1.41, <i>m</i>	27.6 <i>t</i>
16	1.20, <i>m</i> 2.07, <i>m</i>	25.9 <i>t</i>	1.22, <i>m</i> 1.19, <i>m</i>	28.0 <i>t</i>	1.23, <i>m</i> 1.20, <i>m</i>	28.2 <i>t</i>
17	--	44.4 <i>s</i>	--	44.7 <i>s</i>	--	51.3 <i>s</i>
18	2.07, <i>d</i> (3.2)	51.0 <i>d</i>	2.02, <i>brd</i> (3.2)	51.2 <i>d</i>	3.00, <i>d</i> (13.2)	58.2 <i>d</i>
19	1.81, <i>m</i> 1.89, <i>m</i>	37.5 <i>t</i>	2.03, <i>m</i> 1.98, <i>m</i>	39.6 <i>t</i>	1.79, <i>m</i> 1.24, <i>m</i>	44.9 <i>t</i>
20	--	31.6 <i>s</i>	--	31.6 <i>s</i>	--	31.8 <i>s</i>
21	1.45, <i>m</i> 1.31, <i>m</i>	34.4 <i>t</i>	1.48, <i>m</i> 1.39, <i>m</i>	34.1 <i>t</i>	1.55, <i>m</i> 1.42, <i>m</i>	39.8 <i>t</i>
22	1.33, <i>m</i> 1.16, <i>m</i>	30.2 <i>t</i>	2.13, <i>m</i> 1.28, <i>m</i>	21.0 <i>t</i>	4.10, <i>brs</i>	75.8 <i>d</i>
23	1.17, <i>s</i>	26.4 <i>q</i>	1.09, <i>s</i>	26.7 <i>q</i>	1.08, <i>s</i>	26.8 <i>q</i>
24	1.05, <i>s</i>	21.2 <i>q</i>	1.04, <i>s</i>	21.2 <i>q</i>	1.03, <i>s</i>	21.3 <i>q</i>
25	0.98, <i>s</i>	17.4 <i>q</i>	0.98, <i>s</i>	16.2 <i>q</i>	0.97, <i>s</i>	16.4 <i>q</i>
26	1.24, <i>s</i>	19.1 <i>q</i>	1.19, <i>s</i>	18.2 <i>q</i>	1.18, <i>s</i>	18.4 <i>q</i>
27	1.09, <i>s</i>	18.3 <i>q</i>	1.31, <i>s</i>	18.4 <i>q</i>	1.30, <i>s</i>	18.6 <i>q</i>
28	--	179.6 <i>s</i>	--	179.7 <i>s</i>	--	179.9 <i>s</i>
29	0.95, <i>s</i>	33.3 <i>q</i>	0.98, <i>s</i>	33.4 <i>q</i>	0.97, <i>s</i>	33.5 <i>q</i>
30	0.83, <i>s</i>	23.6 <i>q</i>	0.90, <i>s</i>	23.9 <i>q</i>	0.89, <i>s</i>	24.0 <i>s</i>

^a Data for 1 in C₅D₅N while for 2 and 3 in CDCl₃ (400 MHz for ¹H, 100 MHz for ¹³C, δ in ppm).

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Tested Org.	<i>Leishmania donovani</i> Promastigotes		<i>Leishmania donovani</i> Axenic Amastigotes		<i>Leishmania donovani</i> Macrophage -Amastigote		<i>Trypanosoma brucei</i>		THP1 cytotoxicity	
Compounds	IC-50	IC-90	IC-50	IC-90	IC-50	IC-90	IC-50	IC-90	IC-50	IC-90
[1]	>21.3	>21.3	>21.3	>21.3	>21.3	>2.13	5.14± 0.04	6.66± 0.32	>21.3	>2.13
[4]	11.44± 2.11	>22.0	13.90± 3.45	19.47± 1.60	5.74± 3.10	>22.0	0.39± 0.02	1.91± 0.15	16.01±7. 17	19.11±0. 09
[5]	>21.9	>21.9	>21.9	>21.9	>21.9	>21.9	5.67± 0.46	7.20± 0.57	>21.9	>21.9
[6]	>21.2	>21.2	>21.2	>21.2	14.95± 0.42	>21.2	7.12± 0.74	10.51± 4.00	>21.2	>21.2
[10]	>17.6	>17.6	2.54± 0.58	15.15± 1.06	10.89± 3.68	11.90± 4.28	3.20± 0.30	8.13± 1.34	12.57± 2.53	>17.6
Amphotericin B	0.526± 0.216	1.090± 0.254	1.093± 0.794	1.519±0. 573	0.492± 0.200	>10.8	NT	NT	>10.8	>10.8
Pentamidine	2.092±0.4 34	4.866±1. 206	>16.8	>16.8	4.721 ± 1.925	>16.8	0.002 ± 0.0003	0.003 ± 0.0006	>16.8	>16.8
DFMO	NT	NT	NT	NT	NT	NT	11.819± 07300	30.82± 0.659	NT	NT

DFMO, Difluoromethylornithine, NT, Not tested; IC-50 and IC-90 values are expressed as μM and are mean $\pm$ S.D. of duplicate observations.

Tested concentrations range (0.4-10 µg/mL).

Table (3): Antiprotozoal activities of the extracts:

[illegible]

	R ₁	R ₂	R ₃	R ₄
4	O	H	H	H
5	OH	H	H	H
6	OH	OH	H	H
7	O	H	H	Angeloyl
8	O	H	OH	H

Fig.1. Structures of Isolated Metabolites

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HMBC
 ^1H - ^1H COSY

Fig.2 Key HMBC (Arrow) and ^1H - ^1H COSY (Bold Bond) correlations for compounds 1-3

Fig.3 Key ROESY correlations for compounds 2 and 3