21β-Hydroxy-oleanane-type triterpenes from Hippocratea excelsa

David Cáceres-Castillo a, Gonzalo J. Mena-Rejón a, Roberto Cedillo-Rivera b, Leovigildo Quijano c, *

a Laboratorio de Química Orgánica de Investigación, Facultad de Química, Universidad Autónoma de Yucatán, Calle 41 No. 421, Col. Industrial, C.P. 97150 Mérida, Yucatán, Mexico

b Unidad Interinstitucional de Investigación Clínica y Epidemiológica, Facultad de Medicina, UADY/Instituto Mexicano del Seguro Social, Mérida, Yucatán, Mexico

c Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior, Ciudad Universitaria, 04510 México, D.F., México

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Abstract

Stem bark of Hippocratea excelsa afforded six pentacyclic triterpenes, five oleanane and one ursane types. They were identified as 11β,21β-dihydroxy-olean-12-ene-3-one (2), 3α,11α,21β-trihydroxy-olean-12-ene (3), 3α,21β-dihydroxy-11α-methoxy-olean-12-ene (4), 3α,21β-dihydroxy-olean-9(11),12-diene (5), 3α,21β-dihydroxy-olean-12-ene (6) and 3α,21β-dihydroxy-11α-methoxy-urs-12-ene, isolated as its diacetate derivative (7), as well as 3α,21β-dihydroxy-olean-12-ene (1) previously isolated from the root bark. The known α- and β-amyrin, oleanolic and ursolic acids, trans-polyisoprene, and the ubiquitous β-sitosterol were also isolated. Structures were elucidated on the basis of spectroscopic analyses, including homo- and heteronuclear correlation NMR experiments (COSY, ROESY, HSQC and HMBC) and comparison with literature data. The antigiardial activity of compounds 2-5 was not significant.

Keywords: Hippocratea excelsa; Celastraceae; Oleanane triterpenes; Giardia intestinalis

1. Introduction

As part of our continuing search for new biologically active compounds from medicinal plants of the Yucatán Peninsula, we have initiated study of species of the Celastraceae family. Several species of this family have been the subject of continuing interest because of their use in folk medicine and their wide range of biological activities of the isolated metabolites, mainly sesquiterpenes and pentacyclic triterpenoids (Gonzalez et al., 2000).

Hippocratea excelsa H.B.K. of the family Celastraceae is a species with medicinal and insecticidal properties. It is restricted to the tropical deciduous forest of Southern México and Central America (Reyes-Chilpa et al., 2003). The plant is known in Yucatán with the Maya names of “chum-loop”, “salbe’ets”, “sak boób” and “xooknom” (Martínez, 1979). Its root bark is commonly known as “cancerina” and “mata piojo” in the central and southern part of México, because of its use against cancer, lice and mites (Reyes-Chilpa et al., 2003). The plant is also used as an anti-inflammatory and cicatrizing agent, as well as against dysentery, gastritis and gastric ulcers (Palacios et al., 1989; Reyes-Chilpa et al., 2003).

In a previous paper, we described isolation of 21β-hydroxy-β-amyrone (1) from the root bark of H. excelsa, which showed good activity against Giardia intestinalis with an IC50 27.4 μM (Mena-Rejón et al., 2007). In the present paper, we report the results of our chemical study of the stem bark of H. excelsa from Yucatán (México). This resulted in isolation of six new 21β-hydroxy pentacyclic triterpenes, five oleanane and one ursane types, in addition to the previously isolated...
compound 1. All compounds were identified by extensive spectroscopic analyses, and comparison with data reported in the literature. Compounds 2-5 were assayed for their antigiardial activities.

2. Results and discussion

Column chromatography of the hexane and dichloromethane extracts of the dried bark of H. excelsa afforded, in addition to compound 1 isolated before from the roots, six new 21β-hydroxy pentacyclic triterpenes (2-7).

Compound 2 showed an IR spectrum similar to that of compound 1, indicating the presence of hydroxyl and carbonyl groups (3382 and 1696 cm⁻¹). The HR EIMS gave accurate molecular ion peak at m/z 456.3580 corresponding to the molecular formula C₃₀H₄₈O₃ (calcd. 456.3603) which was also in good agreement with the ¹³C NMR spectroscopic data. The ¹H and ¹³C NMR spectra were analogous to that of 1 (Tables 1 and 2), except for the signals associated with the presence of an extra secondary hydroxyl group in the molecule. DEPT experiments confirmed the presence of two hydroxymethine carbons with resonances at δ 74.0 and 81.7 which gave cross-peaks in the HSQC spectrum with proton signals at 3.52 (dd, J = 12.0, 4.8 Hz) and 4.54 (dd, J = 9.6 and 3.2 Hz), respectively.

The chemical shift, coupling constant values and HMBC correlations of the proton signal at 3.52 (δc 74.0) were very similar to those of 1, indicating that one of the hydroxyl groups must be at C-21, and equatorial (β oriented) as deduced by the axial-axial and axial-equatorial couplings of H-21 with H-22 protons, as in compound 1 with a cis fusion and chair-chair conformation of D/E rings (Mena-Rejón et al., 2007). Furthermore, the H-21/H-22 coupling constant values when calculated for the minimum energy conformer of compound 2, using molecular mechanics PC Model version 7.0 with MMX force field (11.0, 4.5 Hz), were in good agreement with the experimental values (12.0, 4.8 Hz).

The multiplicity and coupling constant of the vinyl proton signal (H-12), which appeared at δ 5.50 as a doublet (J = 3.2 Hz), suggested the presence of an allylic hydroxyl group at C-11. The carbon resonances at δC 122.3 and 150.8 confirmed the presence of a Δ¹² double bond, and the oxymethine proton signal at δ 4.54 (dd, J = 9.6 and 3.2 Hz) which correlated with the methine resonance at δ 81.7 in the HSQC spectrum, confirmed that the additional hydroxyl group must be placed at C-11.

Concerning the orientation of the hydroxyl group at C-11, several 11-hydroxy and methoxy Δ¹² oleane and ursane type triterpenes have been reported from different plant families, mainly from the Labiatae (De la Torre et al., 1990; Luis and Andrés, 1999; Bruno et al., 1987; Topcu et al., 2003; Taylor, 1967), Burseraceae (Lima et al., 2004; Lima et al., 2005) and Celastraceae (Muhammad et al., 2000; González et al., 1987; Kutney et al., 1992). In most cases, the orientation of the OH group has been proposed to be in the less hindered equatorial 11α-position, based mainly in the large axial-axial J₁₀,₁₁ values (8-11 Hz). Only three 11β-hydroxy-oleane/ursane type triterpenes from Protium kleinii have been reported recently, the structures being 3-oxo-11β-hydroxy-olean-12-ene (8), 3-oxo-11β-hydroxy-urs-12-ene (9) and 3-oxo-11β,16β-dihydroxy-urs-12-ene (10) (Lima et al., 2005). In this study, the β-orientation of the hydroxyl group at C-11 was established by comparison with the reported ¹³C NMR spectroscopic data for 3-oxo-11α-hydroxy-olean-12-ene (11) (Yuan et al., 1994) and 3-oxo-11α-hydroxy-urs-12-ene (12) (Luis and Andrés, 1999), which showed close similarities except for the large difference for C-11 (δ 67.9 and 68.7 for the reported 11α-hydroxy derivatives, compared with 82.0 and 81.8 for the compounds isolated from P. kleinii) (Lima et al., 2005), as well as differences in the resonances of other neighboring carbon atoms of the C-ring (C-9, C-12 and C-13). Based on these differences, the authors concluded that the compounds isolated from P. kleinii were the 11β-epimers. As a result, it was also suggested that the compound described as 11α-hydroxy-β-amyrin, isolated from Stauntonia hexaphylla callus (Ikuta and Morikawa, 1992) must also be a 11β-hydroxy derivative, since its C-11 carbon...
resonated at δ 81.7. Furthermore the compound isolated from Sabia shumanniana must be the actual 11α-epimer (11), (Yuan et al., 1994). Therefore, the 11-hydroxy-β-amyrin fatty acid ester isolated from Erythroxylum passerinum (Barreiros et al., 2002) must be the 11α-hydroxy derivative instead of the 11β-epimer, as deduced by comparison with the compound isolated by Ikuta and Morikawa (1992). However, the above conclusion seems to be in disagreement with the large J9,11 value (9.4 Hz) for the reported 11β-derivatives from P. kleinii (Lima et al., 2005), although this fact could have an explanation if the dihedral angle between H-11 and H-9 has values close to 0° in the C-11β-hydroxy derivatives. The structure and stereochemistry of 11α-hydroxy-β-amyrin (13) has been determined by X-ray single crystal diffraction but unfortunately no 13C data were given (Kutney et al., 1992).

Comparison of the 13C NMR spectroscopic data of compound 2 from H. excelsa with those reported for the 11β-hydroxy derivatives from P. kleinii (8-10) showed close similarities with exception of the signals associated with the presence of a hydroxyl group instead of the keto group in the molecule. Accordingly, a broad methine singlet appeared at δ 3.42 and the typical signals due to the α-keto methylene were absent in the 1H NMR spectrum. The 13C NMR spectrum exhibited, instead of the carbonyl signal, an extra hydroxymethine signal at δ 76.0, coupled with the broad proton resonance at 3.42 in the HSQC spectrum and the two methyl signals at δ 0.97 (H-23) and 0.87 (H-24) in the HMBC spectrum. In the EIMS, the highest mass peak appeared at m/z 440, which must be due to the loss of H2O and the parent peak at m/z 404 due to the loss of three H2O molecules. Therefore, the structure of compound 3 must correspond to the 2-ketone-reduction product of compound 2. Based on the small equatorial-equatorial and equatorialequatorial couplings of H-3, the hydroxyl group at C-3 must be axially oriented. Comparison of the chemical shift of carbon signals of the A ring with those reported for 3-epi-β-amyrin supported the above assumption. Thus compound 3 was deduced to be 3α,11α,21β-trihydroxy-olean-12-ene (11α,21β-dihydroxy-3-epi-β-amyrin). Another stereoisomer of compound 3, salvinemorol, had been previously isolated from Salvia nemorosa. Its structure was established as 3β,11α,21α-tri-hydroxyolean-12-ene (11β,21α-dihydroxy-β-amyrin) and the stereochemistry at C-21 was based on coupling constants and the inspection of the Dreiding models (Ulubelen et al., 1994), however the model of 11α,21α-dihydroxy-β-amyrin, with a cis D/E ring junction in the chair-chair con-
formation, clearly indicates that H-21 is equatorial and therefore β-oriented and must have equatorial–equatorial and equatorial–axial small coupling values as in yungano-genin C (3β,21α,24-trihydroxy-olean-12-ene) (Ohtani et al., 1992). The reported coupling constants for H-21 for salvinemorol (δH 3.41, dd, J = 11 and 5 Hz; δC 76.0) are similar to those of compound 3 (δH 3.53, dd, J = 11.9, 4.6 Hz; δC 73.9), indicating axial–axial and axial–equatorial interactions of H-21 with H-22. Thus the orientation of H-21 in salvinemorol should be axial (α oriented) and the hydroxyl group β oriented, as in compound 3.

Compound 4, was obtained as colorless amorphous solid. The IR spectrum showed the presence of hydroxyl groups (3413 cm⁻¹/C01) and the absence of carbonyl(s). The ¹H NMR spectrum was very similar to that of 3, with the exception that a signal due to a methoxy group appeared at δ 3.23 and the resonance of H-11 was shifted upfield at δ 3.88 compared with 4.21 in 3. The ¹³C NMR spectrum was also similar to that of 3, except for the presence of one extra carbon signal at δ 53.7 due to the methoxyl group, which was coupled with the resonance at δH 3.88 in the HSQC spectrum, and with the methine at δ 75.9 in the HMBC spectrum. Other differences of 4, when compared with 3 were in the chemical shift of the signals due to C-11 (Δδ +8.3 ppm) and C-9 and C-12 (Δδ −5.2 and −3.6 ppm, respectively) due to β and γ effects of the methyl carbon of the methoxyl group. As in compound 3, the EIMS of 4 did not show a molecular ion peak, but exhibited a highest mass peak at m/z 440 and a parent peak at m/z 404, associated with the loss of MeOH, and two H₂O molecules. Thus compound 4 was identified as 3α,21β-dihydroxy-11α-methoxy-olean-12-ene.

Compound 5, was assigned a molecular formula of C₃₀H₄₈O₂ based on HR EIMS. Its ¹³C NMR spectrum confirmed the presence of 30 carbon atoms due to eight methyl groups, eight methylenes, six methines including two olefinic ones and two hydroxymethines as well as eight quaternary carbons according to DEPT experiments. The ¹H NMR spectrum showed the presence of two vinyl proton signals as an AB pair of doublets at δ 5.54 and 5.61 (J = 5.8 Hz) coupled with the carbon signals at δC 121.4, 115.3, and two protons on hydroxyl-bearing carbons, a

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°, ″ – Interchangeable signals.

a From Mena-Rejón et al. (2007).
b From Lima et al. (2005).
c From Ikuta and Morikawa (1992).
broad singlet at δ 3.42 and a doublet of doublets at δ 3.53 (J = 12.0 and 4.5 Hz), coupled with the resonances at δC 75.7 and 73.9 in the HSQC spectrum. The 1H NMR also confirmed the presence of eight tertiary methyl groups. The EIMS showed a molecular ion (parent peak) at m/z 440 and fragment peaks due to the successive loss of two water molecules at m/z 422 and 404. Based on the above data, compound 5 must the dehydration product of 3, and represent 3α,2β-dihydroxy-olean-9(11),12-diene.

Compound 6, was isolated from the dichloromethane extract. Its IR spectrum showed hydroxyl absorption(s) at 3358 cm⁻¹. The HR EIMS gave a molecular ion peak at m/z 442.3843, corresponding to the molecular formula C₃₀H₅₀O₂ (calcd. 442.3811) which was also in good agreement with the ¹³C NMR and DEPT spectroscopic data. A mass spectral peak at m/z 234 in the EIMS was in agreement with the retro-Diels-Alder fragment of a β-amyrin type triterpene with a hydroxyl group in the E ring as compounds 1–5. The ¹³C NMR spectrum and DEPT experiments of compound 6 indicated the presence of 30 carbon atoms, eight methyl groups, nine methylenes, six methines (including one olefinic and two hydroxymethines), and seven quaternary carbons, one being olefinic. The ¹H NMR spectrum confirmed the presence of eight tertiary methyl groups. It also showed a triplet due to a vinyl proton at δ 5.21 (J = 3.6 Hz), (δC 122.6), and two hydroxymethylene protons (δ 3.40, t, J = 2.4 Hz, δC 76.1 and 3.53, dd, J = 12.0, 4.8 Hz, δC 74.0). The chemical shifts and coupling constants of the hydroxymethylene protons were very similar to those of H-3 and H-21 of compounds 3-5, suggesting that the hydroxyl groups must be at C-3 and C-21, and with the same stereochemistry. Thus compound 6 was identified as, 3α,2β-dihydroxy-olean-12-ene (21β-dihydroxy-3-epi-β-amyrin).

Compound 7, was isolated as the diacetate derivative after acetylation. The ¹H NMR spectrum indicated the presence of one vinyl proton as a doublet at δ 5.43 (J = 3.6 Hz), and three oxygenated methine protons, a triplet of doublets at δ 4.73 (J = 11.2 and 3.8 Hz), a broad singlet at δ 4.61, and a doublet of doublets at δ 3.77 (J = 8.8 and 3.2 Hz). Three sharp singlets at δ 3.30, 2.05 and 2.07 indicated the presence of one methoxyl and two acetyl groups, while the upfield region of the spectrum displayed signals for six tertiary and two secondary methyl groups in the molecule. These data suggested an ursane-type skeleton. The ¹³C NMR spectrum showed signals for 35 carbon atoms due to eleven methyl groups, seven methylenes, nine methines and eight quaternary carbon atoms according with DEPT experiments. Comparison of the ¹H NMR methine signals with those of compound 4 indicated a close relationship. Therefore, the signals at δ 3.77 and 5.43 can be assigned to H-11 and H-12, since they were similar in chemical shifts and coupling constants, while signals at δ 4.61 and 4.73 must correspond to H-3 and H-21 of the carbons bearing the acetoxyl groups, since the signals had shifted downfield, when compared with compound 4. Furthermore, the signal at δ 4.61 showed the same multiplicity, while the signal at δ 4.73 was a triplet of doublets (J = 11.2 and 3.8 Hz), which indicated that H-21 exhibited a further coupling, when compared with compound 4. Therefore, C-20 must be a methane-type carbon bearing a secondary methyl group instead of the gem-dimethyl group. Coupling constant values suggest that H-20 and H-21 must have an equatorial orientation. Therefore the structure of compound 7 was assigned to be 3α,2β-diacetoxy-11α-methoxy-urs-12-ene.

Salvistamineol, the ursane type isomer of salvinemorol has been isolated from Salvia staminea. Its structure was established as 3β,11β,21α-trihydroxy-urs-12-ene. However, as in case of salvinemorol, the coupling constant values indicated an axial–axial between H-21/H-22 and axial-equatorial interactions between H-21/H-22 and H-21/H-20, thus the structure of salvistamineol should be revised to 3β,11α,21β-trihydroxy-urs-12-ene.

Four of five isolated triterpenes (2–5) were assayed against G. intestinalis (Table 3), and full dose–response curves were then obtained for all the compounds. Obtained results clearly showed that only the previous isolated triterpene 1 could be considered an active compound (moderate activity).

3. Experimental section

3.1. General experimental procedures

NMR spectra were recorded in CDCl₃ on Bruker Avance 400 spectrometer. The chemical shifts are given in δ (ppm) with residual CDCl₃ as internal reference and coupling constants in Hz. EIMS were obtained on a Hewlett-Packard 5970 series II gas chromatograph as injection system. HR EIMS were done on a Micromass VG Autospect Geometrys EBE at 70 eV. Optical rotations were measured in CHCl₃ solutions on a Rudolph Research Autopol IV polarimeter. IR spectra were recorded on KBr disks on a Nicolet Magna 750 Fourier transform IR spectrometer. UV spectra were taken in MeOH using Spectronic Unicam Heλios α spectrophotometer. Precoated TLC silica gel 60 F₂₅₄ aluminium sheets from Sigma–Aldrich were used for thin-layer chromatography (0.25 and 0.5 mm layer thickness for analytical and preparative
Dzununcan, 15 km south of the city of Mérida, in the state of Yucatán, México (N 20°51.8′, W 89°38.4′) and authenticated by Dr. José Salvador Flores-Guido. A voucher specimen (J.S. Flores 12350) has been deposited at the Herbarium “Alfredo Barrera Marín”, Universidad Autónoma de Yucatán (UADY), Mérida, Yucatán, México.

3.2. Plant material

H. excelsa H.B.K. was collected on September, 2003 at Dzununcan, 15 km south of the city of Mérida, in the state of Yucatán, México (N 20°51.8′, W 89°38.4′) and authenticated by Dr. José Salvador Flores-Guido. A voucher specimen (J.S. Flores 12350) has been deposited at the Herbarium “Alfredo Barrera Marín”, Universidad Autónoma de Yucatán (UADY), Mérida, Yucatán, México.

3.3. Extraction and isolation

Dried and ground stems bark of H. excelsa (1.6 kg) were successively extracted in a Soxhlet apparatus with n-hexane and CH2Cl2 affording 33 and 12 g of greenish residues after elimination of the solvents, respectively. The n-hexane residue was dissolved in CH2Cl2 and precipitated with MeOH to afford 5.5 g of trans-polysiprose. The n-hexane–methanol solution was concentrated in vacuo giving 17.7 g of residue. The residue was subjected to silica gel CC (63–200 or 2–25 μm particle size) or Sephadex LH-20 from Sigma–Aldrich.

3.4. 11β,21β-Dihydroxy-olean-12-ene-3-one (2)

Amorphous colorless solid; [α]D20 + 4.9 (EtOH: c 0.1); UV max (EtOH): 212, 276, 285; IR νmax cm−1: 3382, 1031, 2951, 2863, 1386, 1696, 756; for 1H (400 MHz, CDCl3) and 13C NMR (100 MHz, CDCl3) spectroscopic data see Tables 1 and 2; EIMS 70 eV, m/z (rel. int.): 456 [M+]+ (7), 454 (38), 438 (86), 420 (64), 405 (25), 339 (24), 289 (62), 271 (79), 248 (66), 230 (48), 219 (34), 201 (40), 189 (31), 187 (39), 173 (38), 171 (35), 159 (43), 145 (50), 135 (84), 119 (92), 107 (86), 95 (93), 81 (75), 69 (85); HR EIMS [M]+ 456.3580 (calcd. C30H48O3, 456.3603).

3.5. 3α,11β,21β-Trihydroxy-olean-12-ene-12 (3)

Amorphous colorless solid; [α]D20 + 6.4 (EtOH: c 0.13); IR νmax cm−1: 3355, 2928, 2861, 1394, 1459, 1034; for 1H (400 MHz, CDCl3) and 13C NMR (100 MHz, CDCl3) spectroscopic data see Tables 1 and 2; EIMS 70 eV, m/z (rel. int.): 440 [M+−18] (13), 422 (12), 405 (32), 404 (100), 390 (16), 389 (52), 323 (17), 253 (40), 213 (14), 201 (22), 199 (16), 187 (19), 173 (17), 159 (18), 147 (15), 145 (22), 135 (16), 133 (24), 123 (19), 121 (25), 119 (31), 109 (25), 95 (33); HR EIMS [M]+−18 440.3626 (calcd. C30H48O2, 440.3654).

3.6. 3α,21β-Dihydroxy-11α-methoxy-olean-12-ene (4)

Amorphous colorless solid; [α]D20 + 7.2 (EtOH: c 0.05); UV max (EtOH): 208, 275 nm; IR νmax cm−1: 3413, 1072, 2950, 2875, 1385, 1460, 756 cm−1; for 1H (400 MHz, CDCl3) and 13C NMR (100 MHz, CDCl3) spectroscopic data see Tables 1 and 2; EIMS 70 eV, m/z (rel. int.): 472 [M]+ (1), 456 (2), 440(8), 405 (31), 404 (100), 390 (14), 389 (50), 384 (12), 253 (35), 213 (14), 202 (11), 201 (21), 199 (14), 189 (13), 187 (20), 173 (19), 173 (13), 159 (19), 145 (21), 135 (14), 133 (24), 123 (17), 121 (20), 119 (28), 109 (25), 95 (30); HR EIMS [M]+ 472.3672 (calcd. C31H52O3, 472.3654).

3.7. 3α,21β-Dihydroxy-olean-9(11),12-diene (5)

Amorphous colorless solid; [α]D20 + 23.1 (MeOH, c 0.12); UV max (EtOH) nm: 208, 275 nm; IR νmax cm−1: 3378, 2930, 2860, 1377, 1461, 1075; for 1H (400 MHz, CDCl3) and 13C NMR (100 MHz, CDCl3) spectroscopic data, see Tables 1 and 2; EIMS 70 eV, m/z (rel. int.): [M]++ (100), 422 (30), 407 (12), 404 (19), 271 (25), 253 (11), 133 (12), 119 (14), 95 (16), 69 (16); HR EIMS 440.3653 (calcd. C30H48O2, 440.3654).
3.8. 3x,21β-Dihydroxy-olean-12-ene (6)

Amorphous colorless solid; [z]$_{D}^{20}$ + 7.6 (EtOH, c 0.04); IR $\nu_{\text{max}}$ cm$^{-1}$: 3358, 2910, 2870, 1394, 1459; for $^{1}$H NMR (400 MHz, CDCl$_3$) and $^{13}$C NMR (100 MHz, CDCl$_3$) spectroscopic data, see Tables 1 and 2; EIMS 70 eV, m/z (rel. int.): 442 [M$^+$] (8), 424 (8), 234 (100), 219 (33), 216 (30), 190 (32), 175 (32), 135 (23), 124 (15), 95 (14); HR EIMS [M$^+$] 442.3834 (calcd. C$_{30}$H$_{50}$O$_2$, 442.3811).

190 (32), 175 (32), 135 (23), 124 (15), 95 (14); HR EIMS [M$^+$] 442.3834 (calcd. C$_{30}$H$_{50}$O$_2$, 442.3811).

3.9. 3x,21β-Diacetoxy-11x-methoxy-urs-12-ene (7)

Amorphous white solid; [z]$_{D}^{20}$ − 1.0 (MeOH, c 0.05); UV $\lambda_{\text{max}}$ nm: 224, 282; IR $\nu_{\text{max}}$ cm$^{-1}$: 2934, 2865, 1732, 1459, 1379, 1246; for $^{1}$H (400 MHz, CDCl$_3$) and $^{13}$C NMR (100 MHz, CDCl$_3$) spectroscopic data, see Tables 1 and 2; EIMS 70 eV, m/z (rel. int.): 556 [M$^+$] (85), 524 (76), 464 (16), 440 (5), 322 (25), 306 (100), 293 (21), 218 (21), 175 (32), 135 (46), 95 (48), 69 (72); HR EIMS 556.4147 (calcd. C$_{35}$H$_{56}$O$_5$, 556.4128).

3.10. Antiprotozoal assay

G. intestinalis IMSS:0696:1 isolated from an individual with symptomatic giardiasis, was used (Cedillo-Rivera et al., 2003). Trophozoites were cultured in TYI-S-33 modified medium, supplemented with 10% calf serum, and subcultured twice a week; for the assay, trophozoites were tested in their log phase of growth (Cedillo-Rivera et al., 1991). In vitro susceptibility assays were developed using a method previously described (Cedillo-Rivera et al., 1992). The compounds (1 mg) dissolved in 1 ml of dimethylsulfoxide (DMSO) were added to Eppendorf tubes containing 1.5 ml of medium in order to get concentrations 1.6, 3.3, 6.6, 13.3 $\mu$g/ml. This solution was inoculated with G. intestinalis to achieve an inoculum of 5 x 10$^4$ trophozoites/ml. Metronidazol was used as the reference drug, culture medium with trophozoites and DMSO was the control, and culture medium was the blank. Inoculated solutions were incubated for 48 h at 37 $^\circ$C. Thereafter, parasites were detached by chilling and trophozoites were counted with a haemocytometer. Experiments were carried out using duplicate tubes and were repeated three times. The 50% inhibitory concentrations (IC$_{50}$), and confidence limits (95%) were calculated by Probit analyses.

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