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Anti-Infective and Cytotoxic Compounds Present in *Blepharodon nitidum*

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Key words
- *Blepharodon nitidum*
- Asclepiadaceae
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- cytotoxic

Abstract

A pharmacological screening of the ethanol extract and fractions of *Blepharodon nitidum* led to the isolation of fourteen compounds, two of which, 24-hydroperoxycycloart-25-en-3β-ol and 25-hydroperoxycycloart-23-en-3β-ol, exhibited *in vitro* anti-*Mycobacterium tuberculosis* and antileishmanial activities, as well as significant cytotoxic activity against a panel of human tumor cell lines.

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Bibliography
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Introduction

In our continuous search for bioactive natural products derived from the Peruvian rainforest [1], [2], [3], we investigated the ethanol extract of *Blepharodon nitidum* (Vell.) J.F. Macbr. (Asclepiadaceae). Although the genus *Blepharodon* contains over seventy species; to the best of our knowledge, there have been no previous pharmacological or phytochemical reports on this genus. *B. nitidum* is a plant not used in Peruvian traditional medicine but *in vitro* screening of its ethanol extract yielded substantial cytotoxic activity. Bioactivity-guided fractionation using silica gel and C18 column chromatography, and C18 HPLC led to the isolation and identification of fourteen compounds, **1 – 14** (**Fig. 1**). The cytotoxic activity against a panel of seven cancer cell lines and two non-tumorogenic cell lines (see **Table 1**) indicated that hydroperoxycycloartanes **11** and **12** are the major anti-cancer principles in *B. nitidum*. Hydroperoxycycloartanes were previously reported to have antibacterial [4] and cytotoxic [5] activity. Because of our increasing interest in the so-called neglected diseases (tuberculosis, malaria, leishmania, and Chagas) that affect large segments of the population in the Third World, we began a screening program in parallel to our anticancer work. Herein we report that compounds **11** and **12** also exhibited significant activities against the axenic amastigotes of *Leishmania amazonensis*, and compound **12** showed moderate activity against a multidrug-resistant strain of *Mycobacterium tuberculosis* (see **Table 2**).

Materials and Methods

Plant material

B. nitidum whole plant was collected in the community of Aramango, province of Bagua, Amazonas Region, Peru, in October 1998. The plant material was identified by one of us (W. H. L.). A voucher specimen under the accession number 19 499 was deposited at the Museo de Historia Natural, Universidad Nacional Mayor de San Marcos, Lima, Peru, and at the Missouri Botanical Garden, St. Louis, MO, USA.

Chemicals and equipment

Column chromatography was carried out using silica gel (230 – 400 mesh, Silicycle) and octadecyl-functionalized silica gel (C18, 200 – 400 mesh, Sigma Aldrich). Reversed-phase HPLC was performed with a Waters 600E equipped with rheodyne injector and a PDA detector, using an analytical column C_{18} Symmetry (Waters, p. s. 5 μm, 4.6 × 250 mm, flow rate 0.9 mL/min; Waters), and a preparative column C_{18} Symmetry (Waters, p. s. 7 μm, 19 × 300 mm, flow rate 9.0 mL/min; Waters). The positive control fludarabine phosphate was purchased from Schering S.p.A while isoniazid and amphotericin B were from Sigma

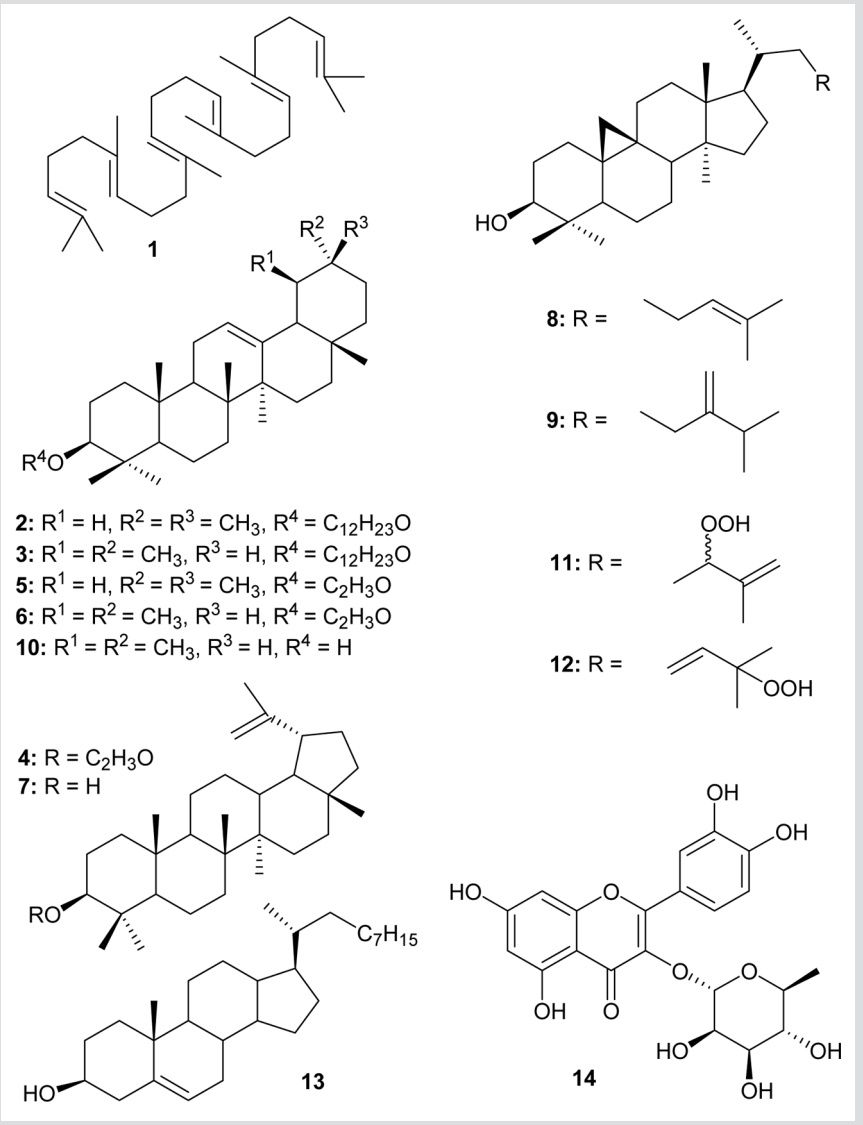

Fig. 1 Chemical structures of metabolites **1 – 14** from *Blepharodon nitidum*.

Aldrich. Fractions were monitored using TLC on silica gel 60 F_{254} (Merck). TLC spots were visualized under UV light and after submersion in 5% sulphuric acid or in 4% phosphomolybdic acid and heating at 120 °C for 2 min. The structures of the isolated compounds were identified by ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), and 2D-NMR analysis in $CDCl_3$ (**1 – 13**) or CD_4OD (**14**) in a Varian Inova 500 (Varian); by GC-EI/MS analysis in a Varian Saturn 2000 (Varian), by MALDI-TOF analysis in a Perspective Biosystems (Perspective Biosystems) using 4-hydroxy-α-cyanocinnamic acid (Aldrich) in EtOH as matrix [4] and by comparison with spectroscopic data with those reported in the literature [3], [4], [5], [6], [7], [8], [9], [10], [11], [12]. The purity of the tested compounds was 80.13% (**11**) and 91.52% (**12**) as determined by analytical HPLC with PDA detection. Copies of the original spectra are obtainable from the author of correspondence.

Extraction and isolation

Air-dried and grounded plant material (50 g) was extracted by percolation with ethanol 95% at room temperature (2 × 1 L, after 3 and 4 days). The ethanolic solution was concentrated to dryness under reduced pressure to yield 5.3 g of extract. An aliquot (4 g) was subjected to column chromatography using 30 g of silica gel (40 – 63 μm; 5 × 4 cm; Silicycle) and a $CHCl_3$-MeOH step gradient (1 : 0, 95 : 5, 9 : 1, 8 : 2, 7 : 3, 6 : 4, 0 : 1; 240 mL each), obtaining 42 fractions (40 mL each), which were analyzed by TLC ($CHCl_3$:MeOH 1 : 0, 20 : 1, 9 : 1, 4 : 1) and grouped into 5 subfractions: A (684 mg), B (85 mg), C (1.2 g), D (823 mg) and E (807 mg). The only cytotoxic fraction (A, GI_{50} > 62 μg/mL) was subjected to chromatography on 60 g of silica gel (40 – 63 μm; 3 × 21.6 cm; Silicycle) using a hexane-$CHCl_3$ step gradient (1 : 0, 60 : 1, 40 : 1, 20 : 1, 10 : 1, 9 : 1, 6 : 1, 5 : 1, 3 : 1, 2 : 1, 1 : 1, 1 : 2, 1 : 10, 1 : 50, 0 : 1; 150 mL each), obtaining 223 subfractions (10 mL each) which were analyzed by TLC (hexanes:$CHCl_3$ 4 : 1, 2 : 1 and $CHCl_3$:MeOH 9 : 1) and grouped into nine subfractions (A1 – A9). Reverse-phase HPLC of fraction A1 (70 mg) using MeOH/2-propanol (3 : 1, λ = 204 nm) yielded squalene (**1**) [7] (1.9 mg, R_t = 31 min). Fraction A3 (30 mg) was eluted using MeOH/2-propanol (45 : 55, λ = 204 nm) yielding β-amyrin laurate (**2**) [8] (1.2 mg, R_t = 67 min) and α-amyrin laurate (**3**) [8] (5.4 mg, R_t = 74 min). An aliquot (70 mg) of fraction A5 was eluted using MeOH/2-propanol (92 : 8, λ = 209 nm) yielding lupeol acetate (**4**) [9] (13 mg, R_t = 32 min), β-amyrin acetate (**5**) [10] (6.8 mg, R_t = 39 min) and α-amyrin acetate (**6**) [10] (24.5 mg, R_t = 43 min). An aliquot (137 mg) of fraction A7 was eluted using MeOH (λ = 204 nm) yielding lupeol (**7**) [9] (35 mg, R_t = 39 min), cycloar-

Table 1 Cytotoxic activity of compounds **11** and **12** from *Blepharodon nitidum* (GI_{50} values μg/mL)[a]

Compound	3T3	H460	DU145	MCF-7	M-14	HT-29	PC3	K562	VERO
11	> 62.5	34.9	> 62.5	> 62.5	33.6	> 62.5	> 62.5	34.4	53.4
12	37.9	30.9	36.1	> 62.5	26.9	29.9	> 62.5	29.9	> 62.5
Fludarabine phosphate	0.00017	> 0.006	0.00026	> 0.0058	0.00033	> 0.0058	0.0041	0.00029	0.00005

[a] The maximum concentration investigated was 62.5 μg/mL.

[b] 3T3, BALB/3T3 clone A31 embryonic mouse fibroblast cells; H460, human large cell lung cancer; DU145, human prostate carcinoma; MCF-7, human breast adenocarcinoma; M-14, human melanoma; HT-29, human colon adenocarcinoma; PC3, human prostate adenocarcinoma; K562, human chronic myelogenous leukemia cells; VERO, normal African green monkey kidney epithelial cells.

Compound	Mycobacterium tuberculosis		Leishmania amazonensis	
	$H_{37}Rv$	MDR	Amastigotes	Macrophages
11	> 25	25	2.4	3.6
12	> 25	12.5	2.3	ND[c]
INH[a]	0.125	4	–	–
ANF[b]	–	–	0.1	5.4

Table 2 Antimycobacterial (MIC μg/mL) and antiprotozoal (IC_{50} μg/mL) activity of compounds **11** and **12**

[a] Isoniazid.

[b] Amphotericin B.

[c] Not determined.

tenol (**8**) [11] (24 mg, R_t = 51 min), 24-methylenecycloartanol (**9**) [12] (9.5 mg, R_t = 55 min), and α-amyrin (**10**) [10] (8.6 mg, R_t = 60 min). Fraction A9 (40 mg) was first eluted with MeOH (λ = 212 nm) to remove green pigments yielding 4 subfractions, the least polar of which (25 mg, R_t = 12 min) was further eluted with H_2O/MeOH/2-propanol (12 : 73 : 15, λ = 204 nm) yielding 24-hydroperoxycycloart-25-en-3β-ol (**11**) [13] (5.4 mg, R_t = 39 min) and 25-hydroperoxycycloart-23-en-3β-ol (**12**) [13] (10.7 mg, R_t = 43 min).

Fraction B was subjected to reverse HPLC using MeOH (λ = 204 nm) as mobile phase yielding β-sitosterol (**13**) [14] (2 mg, R_t = 40 min). Fraction C was subjected to reverse phase column chromatography using 40 g of C18 (40 – 63 μm; 13.5 × 1.5 cm; Sigma Aldrich) using H_2O-MeOH step gradient (6 : 4, 5 : 5, 4 : 6, 3 : 7, 2 : 8, 1 : 9, 0 : 1; 200 mL each) as mobile phase. The initial 15 fractions (100 mL each) were combined into 8 subfractions; an aliquot of the most polar (70 mg) was subjected to reverse HPLC using H_2O/MeOH (55 : 45, λ = 254, 394 nm) as mobile phase yielding quercitrin (**14**) [15] (32 mg, R_t = 25 min).

Cell growth inhibition assay

The percent inhibition of cell growth relative to control of the fractions or pure compounds was evaluated colorimetrically using a sulforhodamine B dye by comparison with the control after incubation in 96-well plates according to a published procedure [16].

Other bioassays

Antimycobacterial experiments were conducted on sensitive $H_{37}Rv$ ATCC 27 294 (American Type Culture Collection) and multidrug-resistant (clinical isolate, strain 02TBDM039EP097) *Mycobacterium tuberculosis* strains [2], [17], while antileishmanial activities were evaluated on axenic amastigotes of *Leishmania amazonensis* (strain MHOM/BR/76/LTB-012) and peritoneal macrophages using previously reported methodologies [18], [19].

Supporting information

^{13}C-NMR data for compounds **1 – 14** and ^{1}H- and ^{13}C-NMR spectra of compounds **11** and **12** are available as Supporting Information.

Results and Discussion

Compounds **1 – 14** were first reported for the *Blepharodon* genus. The anti-cancer, antituberculosis and leishmanicidal activity of hydroperoxycycloartanes **11** and **12** against a panel of seven cancer cell lines and two non-tumorogenic cell lines, sensitive $H_{37}Rv$ and multidrug resistant (MDR) *M. tuberculosis*, and axenic amastigotes of *L. amazonensis* are shown in **Table 1** and **Table 2**. Compounds **11** and **12**, which may be biosynthesized by photooxygenation from the naturally abundant cycloartenol (**8**), have been rarely isolated from plants. Compound **12** exhibited moderate but comparatively higher cytotoxicity than **11**, which has been described as an apoptosis inducer and as a P-gp inhibitor in cancer cells [20]. Both compounds showed selectivity against human cancer cell lines, H460 (large cell lung), M-14 (melanoma) and K562 (chronic myelogenous leukemia cells). Compounds **11** and **12** did not possess significant activity against a sensitive $H_{37}Rv$ *M. tuberculosis* strain at 25 μg/mL, Interestingly, **12** showed moderate activity (12.5 μg/mL) against the MDR *M. tuberculosis* strain. These two tested compounds showed high leishmanicidal activity (2.4 and 2.3 μg/mL respectively). The anti-infective and cytotoxic activities of these hydroperoxycycloartanes might be due to the hydroperoxy group. These two compounds may be regarded as reactive oxygen species (ROS) lipid hydroperoxides (considering the lipophilic saturated cycloartane group) or as producers of ROS, such as superoxide anion, hydroxyl radical and alkoxy radical. Either way, they can potentially damage the microorganisms causing oxidative stress by lipid peroxidation, protein oxidation, or nucleic acid damage. We are contemplating pursuing structure modifications on these and other bioactive isolates.

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References

1 *Moura-Letts G, Villegas LF, Marçalo A, Vaisberg AJ, Hammond GB. In Vivo* wound-healing activity of oleanolic acid derived from the acid hydrolysis of *Anredera diffusa.* J Nat Prod 2006; 69: 978 – 9
2 *Rojas R, Caviedes L, Aponte JC, Vaisberg AJ, Lewis WH, Lamas G et al.* Aegicerin, the first oleanane triterpene with wide-ranging antimycobacterial activity, isolated from *Clavija procera.* J Nat Prod 2006; 69: 845 – 6
3 *Neto CC, Vaisberg AJ, Zhou B-N, Kingston DGI, Hammond GB.* Cytotoxic triterpene acids from the Peruvian medicinal plant *Polylepis racemosa.* Planta Med 2000; 66: 483 – 4
4 *Kato T, Frei B, Heinrich M, Sticher O.* Antibacterial hydroperoxysterols from *Xanthosoma robustum.* Phytochemistry 1996; 41: 1191 – 5
5 *Lee WB, Kwon HC, Cho OR, Lee KC, Choi SU, Baek NI et al.* Phytochemical constituents of *Cirsium setidens* Nakai and their cytotoxic against human cancer cell lines. Arch Pharm Res 2002; 25: 628 – 35
6 *Wang J, Sporns P.* MALDI-TOF MS analysis of food flavonol glycosides. J Agric Food Chem 2000; 48: 1657 – 62
7 *Jautelat M, Grutzner JB, Roberts JD.* Natural-abundance carbon-13 nuclear magnetic resonance spectra of terpenes and carotenes. Proc Natl Acad Sci USA 1970; 65: 288 – 92
8 *Mallavadhani UV, Mahapatra A, Jamil K, Reddy PS.* Antimicrobial activity of some pentacyclic triterpenes and their synthesized 3-*O*-lipophilic chains. Biol Pharm Bull 2004; 27: 1576 – 9
9 *Mahato S, Kundu A.* ^{13}C NMR spectra of pentacyclic triterpenoids – A compilation and some salient features. Phytochemistry 1994; 37: 1517 – 75
10 *Agrawal PK, Jain DC.* ^{13}C NMR spectroscopy of oleanane triterpenes. Prog NMR Spectrom 1992; 24: 1 – 90.
11 *Nes WD, Koike K, Jia Z, Sakamoto Y, Satou T, Nikaido T et al.* 9β,19-Cyclosterol analysis by ^{1}H and ^{13}C NMR, crystallographic observations, and molecular mechanics calculations. J Am Chem Soc 1998; 120: 5970 – 80
12 *Yoshida K, Hirose Y, Imai I, Kondo T.* Conformational analysis of cycloartenol, 24-methylenecycloartanol and their derivatives. Agric Biol Chem 1989; 53: 1901 – 12
13 *Cabrera GM, Seldes AM.* Hydroperoxycycloartanes from *Tillandsia recurvata.* J Nat Prod 1995; 58: 1920 – 4.
14 *De-Eknamkul W, Potduang B.* Biosynthesis of beta-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units. Phytochemistry 2003; 62: 389 – 98
15 *Markham KR.* Carbon-13 NMR studies of flavonoids-III, naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron 1978; 34: 1389 – 97
16 *Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. J Nat Cancer Inst 1990; 4: 1107 – 12.
17 *Caviedes L, Delgado J, Gilman RHJ.* Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis.* Clin Microbiol 2002; 40: 1873 – 4
18 *Castillo D, Arevalo J, Herrera F, Ruiz C, Rojas R, Rengifo E et al.* Spirolactone iridoids might be responsible for the antileishmanial activity of a Peruvian traditional remedy made with *Himatanthus sucuuba* (Apocynaceae). J Ethnopharmacol 2007; 112: 410 – 4.
19 *Sereno D, Lemesre JL.* Axenically cultured amastigote forms as an *in vitro* model for investigation of antileishmanial agents. Antimicrob Agents Chemother 1997; 41: 972 – 6
20 *Madureira AM, Spengler G, Molnár A, Varga A, Molnár J, Abreu PM.* Effect of cycloartanes on reversal of multidrug resistance and apoptosis induction on mouse lymphoma cells. Anticancer Res 2004; 24: 859 – 64