Inhibition of NO Production by *Grindelia argentina* and Isolation of Three New Cytotoxic Saponins

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A bioassay-guided phytochemical analysis of the ethanolic extract of *Grindelia argentina* Deble & Oliveira-Deble (Asteraceae) allowed the isolation of a known flavone, hispidulin, and three new oleanane-type saponins, 3-*O*-*β*-D-xylopyranosyl-(1→3)-*β*-D-glucopyranosyl-2*β*,3*β*,16*α*,23-tetrahydroxyolean-12-en-28-oic acid 28-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-apiofuranosyl-(1→3)-*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl ester (**2**), 3-*O*-*β*-D-glucopyranosyl-2*β*,3*β*,23-trihydroxyolean-12-en-28-oic acid 28-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-apiofuranosyl-(1→3)-*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl ester, (**3**) and 3-*O*-*β*-D-xylopyranosyl-(1→3)-*β*-D-glucopyranosyl-2*β*,3*β*,23-trihydroxyolean-12-en-28-oic acid 28-*O*-*β*-D-xylopyranosyl-(1→2)-*β*-D-apiofuranosyl-(1→3)-*β*-D-xylopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-*α*-L-arabinopyranosyl ester (**4**), named grindeliosides A–C, respectively. Their structures were determined by extensive 1D- and 2D-NMR experiments along with mass spectrometry and chemical evidence. The isolated compounds were evaluated for their inhibitory activities against LPS/IFN-*γ*-induced NO production in RAW 264.7 macrophages and for their cytotoxic activities against the human leukemic cell line CCRF-CEM and MRC-5 lung fibroblasts. Hispidulin markedly reduced LPS/IFN-*γ*-induced NO production (IC_{50} 51.4 μM), while grindeliosides A–C were found to be cytotoxic, with grindelioside C being the most active against both CCRF-CEM (IC_{50} 4.2±0.1 μM) and MRC-5 (IC_{50} 4.5±0.1 μM) cell lines.

1. Introduction. – The genus *Grindelia* (Asteraceae) is represented in South America by 28 species, 25 of them being endemic. Plants from this genus are known to be source of bioactive compounds, mostly diterpenoids of the labdane type [1–8] and manoyl oxide derivatives [9][10], mono- and sesquiterpenes [11–14], polyacetylenes [11], flavonoids [3][5][15], and saponins [16]. Anti-inflammatory, expectorant, antispasmodic, and antimicrobial activities, as well as antifeedant effects towards insects, have been reported for extracts or secondary metabolites obtained from *Grindelia* plants [10][15][17–22].

In the course of our ongoing study of bioactive constituents from native plants from Argentina, we have investigated the EtOH extract of *Grindelia argentina* Deble &

OLIVEIRA-DEBLE (Asteraceae). *G. argentina* is an endemic species growing wildly in Sierra de la Ventana, south of Buenos Aires province, Argentina, on upper stony grasslands [23]. No phytochemical analysis or bioactivity study of *G. argentina* has been conducted so far. A bioassay-guided approach was applied to identify the active secondary metabolites present in the extract that had displayed *in vitro* inhibitory activity on LPS/IFN-γ-induced nitric oxide (NO) production in a preliminary assay. Herein, we describe the isolation and structure elucidation of three new saponins, obtained from the active fractions of the polar sub-extract, and of hispidulin, a known flavone, obtained from the active fractions of the nonpolar sub-extract. Bioactivity results obtained with the extracts, fractions, and isolated compounds for the inhibition of the NO production *in vitro* in LPS/IFN-γ-induced RAW264.7 cells and for cytotoxic activity in the human leukemic cell line CCRF-CEM as well as in MRC-5 lung fibroblasts are also discussed.

2. Results and Discussion. – 2.1. *Extraction and Isolation.* The EtOH extract of *G. argentina* (E) inhibited the LPS/IFN-γ-induced NO production (*Table 1*), and it was partitioned with CH_2Cl_2, BuOH, and H_2O, affording two active sub-extracts, E_1 (CH_2Cl_2) and E_2 (BuOH). Then, E_1 was submitted to vacuum column chromatography (VCC) on silica gel and nine fractions, $E_{1.A}$–$E_{1.I}$, were obtained. The major constituent of the active fraction $E_{1.F}$ was isolated by *SPE-C_{18}* column chromatography (CC) and semi-preparative reversed-phase HPLC. On the basis of its ^{1}H- and ^{13}C-NMR spectra, the isolated compound was identified as the flavone hispidulin (**1**) [24].

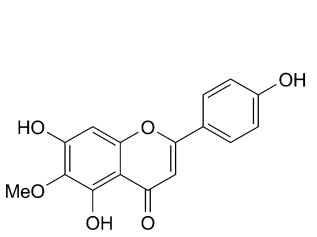
Table 1. *Inhibition of NO Production in LPS-INF-γ Activated RAW264.7 Macrophages by* G. argentina *Extracts, Fractions, and Compounds* **1**–**4**

Inhibitor	Inhibition of NO production[a]) (mean ± SD) [%]
E	51.1 ± 8.2
E_1	69.0 ± 8.4
$E_{1.F}$	81.9 ± 8.9
$E_{2.D}$–$E_{2.E}$	84.6 ± 5.0
1	98.7 ± 0.3
2	89.7 ± 7.1
3	76.4 ± 8.6
4	79.7 ± 6.5
L-NMMA[b])	50.8 ± 4.0[c])

[a]) At 50 μg/ml. [b]) Positive control. [c]) 100 μM.

The BuOH sub-extract E_2 was subjected to CC on *Sephadex LH-20*, and 18 fractions ($E_{2.A}$–$E_{2.R}$) were obtained, among them the active fractions $E_{2.D}$ and $E_{2.E}$, which were rich in saponins. Considering the NO production inhibition displayed by these fractions, we decided to submit them to chromatographic separation. The isolation and purification of the new saponins **2**–**4** was achieved by reversed-phase VCC and repeated semi-preparative HPLC separations.

2.2. *Structure Elucidation of Compounds* **2**–**4**. Compound **2** was obtained as a white amorphous powder with the molecular formula $C_{67}H_{108}O_{35}$, deduced from HR-ESI-MS



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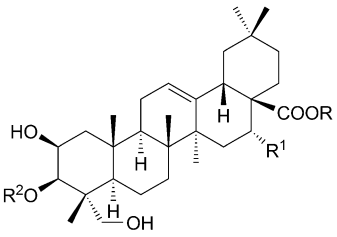

	R^1	R^2
2	OH	Xyl-(1 → 3)-Glc
3	H	Glc
4	H	Xyl-(1 → 3)-Glc

R = Xyl-(1 → 2)-Api-(1 → 2)-Xyl-(1 → 3)-Rha-(1 → 2)-Ara

analysis (*m*/*z* 1495.6656 ([*M*+Na]$^+$)). Ion-trap MS analysis in the negative-ion-mode ESI showed indicative *pseudo*-molecular-ion peaks and MS^n fragment-ion peaks at *m*/*z* 1472 ([*M*−H]$^-$; full MS; parent ion), 1340 ([*M*−H−132]$^-$; MS^2; loss of one pentosyl residue), 1207 ([*M*−H−132−132]$^-$; MS^3; loss of two pentoses), and 797 ([*M*−H−4×132−146]$^-$; MS^3; loss of four pentoses and one desoxyhexose). The ^{13}C-NMR spectrum (*Tables 2* and *3*) showed 67 signals, with 30 attributed to the aglycone portion and 37 to seven monosaccharide moieties. The aglycone presented signals for six Me groups at δ(C) 15.5, 17.8, 18.0, 25.2, 27.6, and 33.6, four CH groups at δ(C) 65.5, 71.3, 74.4, and 83.3, two sp^2 C-atoms at δ(C) 123.6 and 144.5, and one ester C=O group at δ(C) 176.3. These signals indicated the presence of a polygalacic acid moiety with substitutents at C(3) and C(28) [25]. The ^{1}H-NMR spectrum exhibited signals of seven anomeric H-atoms at δ(H) 5.17 (*d*, *J*=7.2), 5.21 (*d*, *J*=7.8), 6.54 (br. *s*), 5.71 (br. *s*), 5.09 (os), 6.41 (br. *s*), and 5.09 (os) that correlated with those at δ(C) 105.9, 106.7, 93.8, 101.6, 106.9, 109.8, and 105.6, respectively, in the HSQC spectrum (*Table 3*). The ^{1}H-NMR CH_2 resonances at δ(H) 3.39 (*t*, *J*=10.8), 3.66 (*d*, *J*=12), and 3.71 (*m*), and the ^{13}C signals respective at δ(C) 67.5, 68.1, and 67.8 were identified as H_a–C(5) and C(5) of three xylose (Xyl) units [26]. The ^{13}C-NMR Me resonance at δ(C) 18.8 (*q*) and CH_2 resonance at δ(C) 62.6 (*t*) allowed the identification of rhamnose (Rha) and glucose (Glc), respectively. The identification of the monosaccharides was completed by the analysis of their anomeric ^{1}H- and ^{13}C-NMR data, coupling constants ^3J(1,2), and GC/MS analysis of the hydrolyzed compound. Their absolute configurations were determined to be D-xylose, D-glucose, L-arabinose, L-rhamnose, and D-apiose by enzymatic hydrolysis, and chemical derivatization [27]. The configuration of the anomeric centers of each sugar was determined by the analysis of the ^1J(C(1),H–C(1)) values observed, leading to α for L-rhamnose (168.0 Hz) and L-arabinose (172.2 Hz) and to β for D-xyloses (160.2, 158.4, and 157.8 Hz), and D-glucose (153.0 Hz) [28]. For D-apiose, the β configuration was assigned by comparison with the spectroscopic data in the literature [29]. In the HMBC spectrum, the correlations of Glc H–C(1) (δ(H) 5.17) with aglycone C(3) (δ(C) 83.3), and Xyl H–C(1) (δ(H) 5.21) with Glc C(3) (δ(C) 88.1) indicated 3-*O*-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl as the substitutent at C(3) of the polygalacic acid moiety (*Fig.*). This spectrum also evidenced the presence of 28-*O*-β-D-xylopyranosyl-(1→2)-β-D-apiofuranosyl-(1→3)-β-D-xylopyranosyl-(1→

3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl based on the correlations of arabinose (Ara) H–C(1) (δ(H) 6.54) with aglycone C(28) (δ(C) 176.3), Rha H–C(1) (δ(H) 5.71) with Ara C(2) (δ(C) 75.9), Xyl-I H–C(1)(δ(H) 5.09) with Rha C(4) (δ(C) 84.1), apiose (Api) H–C(1) (δ(H) 6.41) with Xyl-I C(3) (δ(C) 83.5), and Xyl-II H–C(1) (δ(H) 5.09) with Api C(2)(δ(C) 84.9) (*Fig.*). Then, grindelioside A (**2**) was identified as 3-*O*-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-2β,3β,16α,23-tetrahydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl-(1→2)-β-D-apiofuranosyl-(1→3)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl ester.

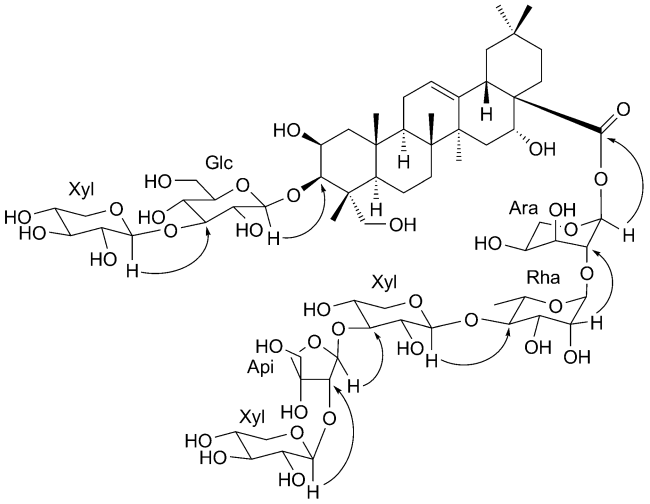

Figure. *Key HMBCs for sugar chains at C(3) and C(28) of* **2**

Compound **3** was obtained as a white amorphous powder with the molecular formula $C_{62}H_{100}O_{30}$, deduced from HR-ESI-MS analysis (*m/z* 1347.6274 ($[M+Na]^+$)). The negative-ion-mode trap mass spectrum showed a *pseudo*-molecular ion at *m/z* 1324 ($[M-H]^-$; parent ion) and 1370 ($[M-H+HCOOH]^-$; formic acid adduct) (full MS). The ^{13}C-NMR spectrum of **3** showed 30 signals attributed to the aglycone together with 32 signals due to six sugar moieties (*Tables 2* and *3*). The ^{1}H-NMR spectrum of **3** exhibited six anomeric signals at δ(H) 5.17 (*d*, *J* = 7.2), 6.52 (*d*, *J* = 2.4), 5.77 (br. *s*), 5.08 (os), 6.44 (br. *s*), and 5.10 (*d*, *J* = 7.8) that correlate with those at δ(C) 106.2, 93.9, 101.5, 107.2, 109.9, and 105.6, respectively, in the HSQC spectrum (*Table 3*). The ^{13}C-NMR resonances due to six Me groups at δ(C) 15.5, 17.7, 18.0, 24.1, 26.5, and 33.5, three CH groups at δ(C) 66.1, 71.0, and 83.5, two sp^2 C-atoms at δ(C) 123.6 and 144.6, and one ester C=O group at δ(C) 176.7 indicated the presence of a bayogenin glycoside with substituents at C(3) and C(28) (*Table 2*) [25]. The nature of the sugars and their absolute configurations were determined as described for **2**. The comparison of ^{1}H- and ^{13}C-NMR spectra of **2** and **3** evidenced the presence of the same monosaccharide sequence attached to aglycone at C(28) for both saponins, and, in the case of **3**, only one glucose unit was attached to C(3). Assignments of all ^{1}H- and

Table 2. *^{1}H- and ^{13}C-NMR Data for Aglycones of* **2**–**4**. In C_5D_5N; δ in ppm, J in Hz.

Position	**2**		**3**		**4**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	2.28 (os)[a]), 1.27 (os)	44.7 (*t*)	2.28 (*dd*, *J* = 13.8, 1.8), 1.24 (os)	44.6 (*t*)	2.25 (*d*, *J* = 13.2), 1.24 (os)	44.7 (*t*)
2	4.81 (br. *s*)	71.3 (*d*)	4.81–4.84 (*m*)	71.0 (*d*)	4.79–4.83 (*m*)	71.2 (*d*)
3	4.36 (os)	83.3 (*d*)	4.35 (os)	83.5 (*d*)	4.36 (os)	83.3 (*d*)
4		42.6 (*s*)		42.2 (*s*)		42.3 (*s*)
5	1.85 (os)	48.2 (*d*)	1.79 (os)	48.1 (*d*)	1.82 (os)	48.1 (*d*)
6	1.80 (os), 1.59 (os)	18.5 (*t*)	1.81 (os), 1.59 (os)	18.4 (*t*)	1.83 (os), 1.60 (os)	18.5 (*t*)
7	1.81 (os), 1.48 (os)	33.6 (*t*)	1.44 (*d*, *J* = 12.0)	33.4 (*t*)	1.46 (os)	33.3 (*t*)
8		40.6 (*s*)		40.5 (*s*)		40.4 (*s*)
9	1.88 (os)	48.1 (*d*)	1.74 (os)	48.9 (*d*)	1.74 (os)	48.9 (*d*)
10		37.4 (*s*)		37.4 (*s*)		37.4 (*s*)
11	2.17 (os), 2.06 (*d*, *J* = 16.8)	24.4 (*t*)	2.09 (os), 1.99 (os)	24.5 (*t*)	2.11 (os), 1.99 (os)	24.4 (*t*)
12	5.65 (br. *s*)	123.6 (*d*)	5.47 (br. *s*)	123.6 (*d*)	5.47 (br. *s*)	123.6 (*d*)
13		144.5 (*s*)		144.6 (*s*)		144.6 (*s*)
14		42.6 (*s*)		42.7 (*s*)		42.7 (*s*)
15	2.35 (os), 1.76 (os)	36.6 (*t*)	2.10 (os), 1.23 (os)	28.7 (*t*)	2.10 (os), 1.23 (os)	28.7 (*t*)
16	5.26 (br. *s*)	74.4 (*d*)	2.08 (os), 1.99 (os)	23.6 (*t*)	2.08 (os), 2.01 (os)	23.6 (*t*)
17		50.0 (*s*)		47.8 (*s*)		47.8 (*s*)
18	3.61 (os)	41.7 (*d*)	3.28 (*dd*, *J* = 13.8, 3.6)	42.1 (*d*)	3.31 (*dd*, *J* = 13.2, 3.6)	42.1 (*d*)
19	2.77 (*dd*, *J* = 13.2, 13.8), 1.37 (os)	47.4 (*t*)	1.77 (os), 1.25 (os)	46.6 (*t*)	1.75 (os), 1.27 (os)	46.6 (*t*)
20		31.3 (*s*)		31.3 (*s*)		31.3 (*s*)
21	2.41 (os), 1.32 (os)	36.4 (*t*)	1.38 (os), 1.17 (os)	34.6 (*t*)	1.39 (os), 1.16 (os)	34.5 (*t*)
22	2.31 (os), 2.23 (os)	32.5 (*t*)	2.02 (os), 1.71 (os)	33.1 (*t*)	2.02 (os), 1.74 (os)	33.2 (*t*)
23	4.36 (os), 3.72 (os)	65.5 (*t*)	4.38 (os), 3.73 (*d*, *J* = 10.2)	66.1 (*t*)	4.37 (os), 3.73 (os)	65.5 (*t*)
24	1.37 (*s*)	15.5 (*q*)	1.39 (*s*)	15.5 (*q*)	1.37 (*s*)	15.5 (*q*)
25	1.59 (*s*)	17.8 (*q*)	1.55 (*s*)	17.7 (*q*)	1.56 (*s*)	17.7 (*q*)
26	1.16 (*s*)	18.0 (*q*)	1.13 (*s*)	18.0 (*q*)	1.14 (*s*)	18.0 (*q*)
27	1.78 (*s*)	27.6 (*q*)	1.24 (*s*)	26.5 (*q*)	1.25 (*s*)	26.5 (*q*)
28		176.3 (*s*)		176.7 (*s*)		176.7 (*s*)
29	1.01 (*s*)	33.6 (*q*)	0.91 (*s*)	33.5 (*q*)	0.91 (*s*)	33.6 (*q*)
30	1.16 (*s*)	25.2 (*q*)	1.00 (*s*)	24.1 (*q*)	1.00 (*s*)	24.1 (*q*)

[a]) os, Overlapped signal.

Table 3. *^{1}H- and ^{13}C-NMR Data for the Sugars of* **2**–**4**. In C_5D_5N; δ in ppm, J in Hz.

Position	**2**		**3**		**4**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
Glc						
1	5.17 (*d*, *J* = 7.2)	105.9 (*d*)	5.17 (*d*, *J* = 7.8)	106.2 (*d*)	5.17 (*d*, *J* = 7.8)	106.0 (*d*)
2	4.05 (os)[a]	74.8 (*d*)	4.05 (os)	75.9 (*d*)	4.05 (os)	74.8 (*d*)
3	4.09 (os)	88.1 (*d*)	4.18 (os)	79.0 (*d*)	4.10 (os)	88.1 (*d*)
4	4.14 (os)	69.8 (*d*)	4.24 (os)	72.0 (*d*)	4.14 (os)	69.8 (*d*)
5	3.86–3.88 (*m*)	78.4 (*d*)	3.91–3.94 (*m*)	78.7 (*d*)	3.85–3.87 (*m*)	78.4 (*d*)
6	4.42 (os), 4.28 (os)	62.6 (*t*)	4.48 (os), 4.35 (os)	63.1 (*t*)	4.42 (os), 4.30 (os)	62.6 (*t*)
Xyl						
1	5.21 (*d*, *J* = 7.8)	106.7 (*d*)			5.22 (*d*, *J* = 7.8)	106.8 (*d*)
2	4.03 (os)	75.7 (*d*)			4.04 (os)	75.8 (*d*)
3	4.17 (os)	78.6 (*d*)			4.17 (os)	78.6 (*d*)
4	4.17 (os)	71.2 (*d*)			4.18 (os)	71.3 (*d*)
5	4.34 (os), 3.71 (os)	67.8 (*t*)			4.33 (os), 3.71 (os)	67.9 (*t*)
Ara						
1	6.54 (br. *s*)	93.8 (*d*)	6.52 (*d*, 2.4)	93.9 (*d*)	6.54 (br. *s*)	93.8 (*d*)
2	4.55 (os)	75.9 (*d*)	4.58 (os)	75.9 (*d*)	4.58 (os)	75.8 (*d*)
3	4.56 (os)	70.0 (*d*)	4.56 (os)	70.2 (*d*)	4.58 (os)	70.1 (*d*)
4	4.44 (os)	66.2 (*d*)	4.44 (os)	66.5 (*d*)	4.44 (os)	66.4 (*d*)
5	4.56 (os), 3.96 (os)	63.1 (*t*)	4.54 (os), 3.96 (*dd*, *J* = 10.8, 3.6)	63.4 (*t*)	4.54 (os), 3.96 (*dd*, *J* = 10.2, 3.6)	63.3 (*t*)
Rha						
1	5.71 (br. *s*)	101.6 (*d*)	5.77 (br. *s*)	101.5 (*d*)	5.76 (br. *s*)	101.5 (*d*)
2	4.56 (os)	72.2 (*d*)	4.59 os	72.2 (*d*)	4.59 os	72.2 (*d*)
3	4.58 (os)	73.1 (*d*)	4.60 os	73.2 (*d*)	4.60 os	73.2 (*d*)
4	4.35 (os)	84.1 (*d*)	4.35 os	84.5 (*d*)	4.37 os	84.4 (*d*)
5	4.41 (os)	69.0 (*d*)	4.42 os	69.0 (*d*)	4.42 os	69.0 (*d*)
6	1.73 (*d*, *J* = 6.0)	18.8 (q)	1.78 (*d*, *J* = 6.0)	18.8 (*q*)	1.78 (*d*, *J* = 6.0)	18.8 (*q*)
Xyl-I						
1	5.09 (os)	106.9 (*d*)	5.08 (os)	107.2 (*d*)	5.08 (*d*, *J* = 7.8)	107.1 (*d*)
2	4.02 (os)	75.7 (*d*)	4.04 (os)	75.8 (*d*)	4.04 (os)	75.8 (*d*)

Table 3 (cont.)

Position	**2**		**3**		**4**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
3	4.12 (os)	83.5 (*d*)	4.14 (os)	83.5 (*d*)	4.14 (os)	83.5 (*d*)
4	4.02 (os)	69.7 (*d*)	4.05 (os)	69.8 (*d*)	4.04 (os)	69.7 (*d*)
5	4.15 (os), 3.39 (*t*, *J* = 10.8)	67.5 (*t*)	4.16 (os), 3.41 (*t*, *J* = 10.8)	67.6 (*t*)	4.18 (os), 3.41 (*t*, *J* = 10.8)	67.6 (*t*)
Api						
1	6.41 (br. *s*)	109.8 (*d*)	6.44 (br. *s*)	109.9 (*d*)	6.44 (br. *s*)	109.8 (*d*)
2	5.04 (os)	84.9 (*d*)	5.06 (br. *s*)	84.9 (*d*)	5.06 (br. *s*)	84.9 (*d*)
3		81.8 (*s*)		81.9 (*s*)		81.9 (*s*)
4	4.64 (*d*, *J* = 9.0), 4.20 (os)	76.1 (*t*)	4.64 (*d*, *J* = 9.0), 4.20 (os)	76.2 (*t*)	4.64 (*d*, *J* = 9.0), 4.21 (os)	76.2 (*t*)
5	4.22 (os)	66.3 (*t*)	4.23 (os)	66.3 (*t*)	4.23 (os)	66.3 (*t*)
Xyl-II						
1	5.09 (os)	105.6 (*d*)	5.10 (*d*, *J* = 7.8)	105.6 (*d*)	5.11 (*d*, *J* = 7.8)	105.6 (*d*)
2	4.52 (os)	73.2 (*d*)	4.51 (os)	73.2 (*d*)	4.51 (os)	73.2 (*d*)
3	4.06 (os)	75.1 (*d*)	4.04 (os)	75.2 (*d*)	4.04 (os)	75.3 (*d*)
4	4.21 (os)	70.2 (*d*)	4.20 (os)	70.3 (*d*)	4.20 (os)	70.2 (*d*)
5	4.25 (os), 3.66 (*d*, *J* = 12.0)	68.1 (*t*)	4.22 (os), 3.66 (*d*, *J* = 11.4)	68.2 (*t*)	4.22 (os), 3.66 (*d*, *J* = 11.4)	68.2 (*t*)

[a]) os, Overlapped signal.

^{13}C-NMR resonances were achieved with the aid of COSY, HSQC, and HMBC experiments, as described for **2** (*Tables 2* and *3*). The α-, α-, β-, and β-configurations for L-rhamnose, L-arabinose, D-glucose, and D-xyloses, respectively, were assigned from their ^1J(C(1),H–C(1)) values (168.6, 172.2, 150.6 and 159.0, and 155.4 Hz, resp.) [28]. Therefore, compound **3**, named grindelioside B, was identified as 3-*O*-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl-(1→2)-β-D-apiofuranosyl-(1→3)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl ester.

The HR-ESI-MS analysis of grindelioside C (**4**) indicated the molecular formula $C_{67}H_{108}O_{34}$, deduced from *m*/*z* 1479.6716 ($[M+Na]^+$)). Ion-trap MS analysis in the negative-ion-mode ESI showed indicative *pseudo*-molecular-ion and MS^n fragment-ion peaks at *m*/*z* 1456 ($[M-H]^-$; parent ion, full MS),1324 ($[M-H-132]^-$; loss of one pentosyl residue, MS^2), 1192 ($[M-H-132-132]^-$; loss of two pentoses, MS^3), and 782 ($[M-H-4\times132-146]^-$; MS^3; loss of four pentoses and one desoxyhexose). The fragmentation pattern of this molecule was the same as for **2**. The presence of seven sugar moieties was indicated by the seven anomeric H-atom signals (δ(H) 5.17, 5.22, 6.54, 5.76, 5.08, 6.44, and 5.11) in the ^{1}H-NMR spectrum, associated to the corresponding anomeric C-atom atoms in the ^{13}C-NMR spectrum (δ(C) 106.0, 106.8, 93.8, 101.5, 107.1, 109.8, and 105.6, resp.) through HSQCs (*Table 3*). The comparison of the molecular formula of **4** with that of **3** suggested the presence of an additional pentose monosaccharide. Furthermore, the resonances of the aglycone moiety of **4** were almost identical to those of **3**, while the signals corresponding to the monosaccharides showed no differences from those observed for compound **2** (*Tables 2* and *3*). This evidence suggested the presence of a bayogenin aglycone substituted at C(3) and C(28) with the same di- and pentasaccharide chains as in **2**. Thus, the structure of **4** has been determined as 3-*O*-β-D-xylopyranosyl-(1→3)-β-D-glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oic acid 28-*O*-β-D-xylopyranosyl-(1→2)-β-D-apiofuranosyl-(1→3)-β-D-xylopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl ester.

2.3. *Biological Assays.* Compounds **1**–**4** were evaluated for their inhibitory activities against NO production in LPS/IFN-γ-activated RAW264.7 cells (*Table 1*).

NO is a gaseous free radical. It is produced from the amino acid L-arginine by three enzymes including inducible nitric oxide synthase (iNOS). In recent years, inhibition of iNOS turned out to be a subject of interest in the field of anti-inflammatory research, as NO produced by iNOS has been shown to be involved in various physiological and pathophysiological conditions. iNOS-Driven overproduction of NO in various cell types seems to be involved in several inflammatory and immunoregulatory processes. Additionally, NO contributes to a number of pathophysiological conditions such as cancer, rheumatoid arthritis, diabetes, liver cirrhosis, septic shock, and cardiovascular diseases [30][31].

All the tested compounds showed strong inhibitory activities against NO production. Compound **1** showed the most potent inhibition with 98.7% at 50 μg/ml concentration (IC_{50} 51.4 μM) and had no effect on cell viability. The inhibitory activities of compounds **2**–**4** resulted to be false-positive, as these compounds turned out to be cytotoxic: grindelioside A (**2**), grindelioside B (**3**), and grindelioside C (**4**) elicited strong cytotoxicity in the XTT (=2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetra-

zolinum-5-carboxanilide) assay in the human leukemic cell line CCRF-CEM (IC_{50} values of 4.2 ± 0.1, 12.4 ± 0.4, and 10.4 ± 0.3 μM, resp.) as well as in MRC-5 lung fibroblasts (IC_{50} values of 4.5 ± 0.1, 13.8 ± 0.6, and 11.1 ± 0.4 μM, resp.).

3. Conclusions. – Several biological activities including antifungal, antiproliferative, antioxidant, antithrombosis, and antitumor properties have been reported for the flavone hispidulin (**1**)[32]. The presence of **1** has been reported in *Artemisia vestita* and *Eupatorium arnottianum*, two medicinal plants used for treatment of inflammatory diseases [33][34]. As far as we know, this is the first report on inhibitory activity of this compound against LPS-induced NO production. Hispidulin (**1**) exhibited an inhibitory activity (IC_{50} 51.47 μM) comparable to other naturally occurring flavones with the same substitution pattern at C(4′), C(5), and C(7) [35–38].

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Experimental Part

General. Column chromatography (CC): silica gel *60* (SiO_2; 40–63 μm; *Merck*), octadecyl-functionalized SiO_2 (*LiChroPrep RP-18*, 40–63 μm; *Merck*), and *Sephadex LH-20* (*GE-Healthcare*). Anal. TLC: SiO_2 *60* F_{254} sheets (0.2-mm thickness, *Merck*). HPLC: *Agilent 1260 Infinity* system equipped with a diode array detector, *Agilent Zorbax® SB-*C_{18} column (2.1 × 150 mm, 3.5 μm) protected by a *Zorbax® SB-C-8* guard column (2.1 × 12.5 mm; 5 μm). Semi-prep. HPLC: *Varian® PrepStar SD-1* with *Dynamax®* solvent delivery system and a *Dynamax®* absorbance detector model *UV-1*, *UltraSep ES® 100 RP-18* column (250 × 20 mm, 10 μm) or a *LichroSorb® RP-18* (7 μm), or *LiChroCART* cartridge (250 × 10 mm, *Merck*). GC/MS: *Agilent 7890A* apparatus (*7683B Series Autosampler* and *7975C MSD*), *HP5-MS* cap. column (30 m × 250 μm × 0.25 μm, *Agilent*). M.p.: *Reichert* melting points apparatus. Optical rotations: *Polar IBZ Messtechnik* polarimeter. ^{1}H- and ^{13}C-NMR Spectra: *Varian Unitylnova* spectrometer operated at 600 and 150 MHz, resp., δ in ppm rel. to Me_4Si as internal standard, J in Hz. ESI-MS: *LTQ XL* linear ion trap mass spectrometer equipped with an *H-ESI II* probe (*Thermo Scientific*) in the positive- and negative-ion mode (cap. temp., 330°; ion spray voltage, 5 kV; cap voltage, 15 V). HR-ESI-MS: *Waters Synapt HDMS q-TOF* MS detector in the positive-ion mode ESI; in *m*/*z*.

Plant Material. Aerial parts of *G. argentina* were collected (October 2008) in Sierra de la Ventana, Buenos Aires Province, Argentina, and identified by Dr. *Maria Gabriela Murray*. A voucher specimen (MGM 483) was deposited with the Herbario del Departamento de Biología, Bioquímica y Farmacia-Universidad Nacional del Sur (BBB).

Extraction and Isolation. Fresh aerial parts from *G. argentina* (1380 g) were extracted with EtOH (8 l) at r.t. for two weeks and evaporated to dryness to yield 63.0 g of extract (*E*). A portion of this extract (56.7 g) was suspended in H_2O/MeOH 9:1 (1.1 l) and then partitioned with CH_2Cl_2 (3 × 320 ml) to obtain E_1 sub-extract (8.1 g). After evaporation to dryness, it was subjected to vacuum liquid chromatography (VLC; SiO_2 *60*, 230–400 mesh, 100 g), eluting with hexane/AcOEt of increasing polarity, and fractions $E_{1.A}$–$E_{1.I}$ (600 ml each) were obtained. *Fr.* $E_{1.F}$ (330 mg), eluted with hexane/AcOEt 50:50, was subjected to CC (*SPE-*C_{18}; MeOH/H_2O) to give fractions $E_{1.F.a}$–$E_{1.F.k}$. *Fr.* $E_{1.F.g}$ (48 mg) was dissolved in 5 ml of HPLC-grade MeCN/MeOH 3:2 and subjected to semi-prep. RP-HPLC (C_{18} column, (*LiChroSorb® RP-18* (7 μm), *LiChroCart* (250 × 10 mm)), MeCN/H_2O 25:75 to 30:70; in 50 min at 5 ml/min; detection wavelength 220 nm) to furnish compound **1** (4.3 mg) identified as hispidulin. The ^{13}C-NMR spectrum recorded in (D_6)DMSO was in agreement with literature data [24].

Another portion of the *E* extract (6.3 g) was suspended in H_2O/MeOH 9:1 (125 ml) and then partitioned with CH_2Cl_2 (3 × 35 ml) and BuOH (3 × 35 ml) to obtain sub-extracts E_1 (0.9 g) and E_2 (0.8 g), resp. The BuOH sub-extract E_2, was subjected to CC (*Sephadex LH-20*; MeOH (450 ml)). Eighteen fractions of 25 ml were collected ($E_{2.A}$–$E_{2.R}$), from which $E_{2.D}$ and $E_{2.E}$ were combined and evaporated to dryness *in vacuo* (293 mg). Then, they were subjected to CC (*RP-18* (30 g); MeOH/H_2O 0:100–100:0) to give 13 fractions of 50 ml ($E_{2.DE.a}$–$E_{2.DE.m}$). *Fr.* $E_{2.DE.g}$ (103.5 mg) was dissolved in 10 ml of HPLC-grade MeCN/H_2O 1:1 and subjected to semi-prep. RP-HPLC (C_{18} column (*Ultra Sep ES 100 RP18*; 250 × 20 mm, 10 μm); MeCN/H_2O 22:78 to 26:74 in 50 min at 10 ml/min; detection wavelength 205 nm and injecting 100 μl) to obtain **2** (t_R 23 min; 29.8 mg). Compounds **3** (t_R 18 min; 8.9 mg) and **4** (t_R 19 min; 10.1 mg) were obtained after HPLC separation of fraction *H* (108 mg) using a gradient MeCN/H_2O 26:74 to 29:71 in 30 min and the same chromatographic conditions as described for **1**. The purity of compounds **2**–**4** was determined by anal. HPLC: pure compounds were dissolved in MeOH (1 mg/ml) and analyzed by injecting 10 μl on an *Agilent Zorbax® SB-C18* column and using the following conditions: solvent *A*, MeCN + 0.1% HCOOH; solvent *B*, H_2O + 0.1% HCOOH; from 10 to 100% of solvent *A* in 50 min; flow rate, 0.25 ml/min.

Grindelioside A (= *β*-D-*Xylopyranosyl-(1→2)-β*-D-*apiofuranosyl-(1→3)-β*-D-*xylopyranosyl-(1→3)-α*-L-*rhamnopyranosyl-(1→2)-α*-L-*arabinopyranosyl 3*-O-*β*-D-*Xylopyranosyl-(1→3)-β*-D-*glucopyranosyl-2β,3β,16α,23-tetrahydroxyolean-12-en-28-oate*; **2**). White solid, soluble in MeOH and pyridine. M.p. 229–231°. $[\alpha]_D^{22} = -34.0$ ($c = 0.25$, pyridine). ^{1}H- and ^{13}C-NMR: *Tables 2* and *3*. ESI-MS (neg.): 1472 ($[M-H]^-$; full MS), 1340 ($[M-H-132]^-$; MS^2), 1207 ($[M-H-132-132]^-$; MS^3), 797 ($[M-H-4\times132-146]^-$; MS^3). HR-ESI-MS (pos.): 1495.6656 ($[M+Na]^+$, $C_{67}H_{108}NaO_{35}^+$; calc. 1495.6569).

Grindelioside B (= *β*-D-*Xylopyranosyl-(1→2)-β*-D-*apiofuranosyl-(1→3)-β*-D-*xylopyranosyl-(1→3)-α*-L-*rhamnopyranosyl-(1→2)-α*-L-*arabinopyranosyl 3*-O-*β*-D-*Glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate*; **3**). White solid, soluble in MeOH and pyridine. M.p. 228–230°. $[\alpha]_D^{22} = -19.1$ ($c = 0.11$, pyridine). ^{1}H- and ^{13}C-NMR: *Tables 2* and *3*. ESI-MS (neg.): 1324 ($[M-H]^-$), 1370 ($[M-H+HCOOH]^-$; full MS). HR-ESI-MS (pos.): 1347.6274 ($[M+Na]^+$, $C_{62}H_{100}NaO_{30}^+$; cal. 1347.6197).

Grindelioside C (= *β*-D-*Xylopyranosyl-(1→2)-β*-D-*apiofuranosyl-(1→3)-β*-D-*xylopyranosyl-(1→3)-α*-L-*rhamnopyranosyl-(1→2)-α*-L-*arabinopyranosyl 3*-O-*β*-D-*Xylopyranosyl-(1→3)-β*-D-*glucopyranosyl-2β,3β,23-trihydroxyolean-12-en-28-oate*; **4**). White solid, soluble in MeOH and pyridine. M.p. 236–238°. $[\alpha]_D^{22} = -20.0$ ($c = 0.18$, pyridine). ^{1}H- and ^{13}C-NMR: *Tables 2* and *3*. ESI-MS (neg.): 1456 ($[M-H]^-$; full MS), 1324 ($[M-H-132]^-$; MS^2), 1192 ($[M-H-132-132]^-$; MS^3), 782 ($[M-H-4\times132-146]^-$; MS^3). HR-ESI-MS (pos.): 1479.6716 ($[M+Na]^+$, $C_{67}H_{108}NaO_{34}^+$; calc. 1479.6620).

Determination of Absolute Configuration of Sugars. Absolute configurations of sugars were deduced as described in [27]. Briefly, *β*-glucuronidase from *Helix pomatia* (*Type H-1*, 3015000 U/g, *Sigma*; 300 μg in 100 μl of H_2O) was added to 300 μg of each saponin in 100 μl of H_2O. The mixture was kept at 37° (drying cabinet) for 7 h in a stoppered reaction vial. After drying the soln. with a stream of N_2, 45 μl of (−)-(*R*)-butan-2-ol and 5 μl of 1N HCl were added to the residue, and the mixture was heated at 100° for 15 h. After evaporation, 50 μl of trimethylsilylation reagent (*Sigma-Sil-A®*, *Sigma*) were added to the dried residue. The reference sugars were treated under the same conditions. All samples were analyzed by GC/MS, and retention times of the hydrolysates of compounds **2**–**4** were compared with those of the authentic samples. Injector and detector temps. were set at 270°, and the oven temp. program was as follows: 100° for 0 min, increased at 3°/min to 270°, and then kept at 270° for 5 min. Samples (1 μl) were injected in the split mode. Mass spectra were acquired over a 40–700 amu with ionizing electron energy of 70 eV. The carrier gas was He at 0.9 ml/min.

Determination of the NO Release: The inhibition of NO production *in vitro* in LPS/IFN-*γ*-induced RAW264.7 cells was determined by the *Griess* assay method, as described in [39]. The NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) was used as positive control.

XTT Viability Assay. The XTT (=2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) viability assay was performed as described previously [40] and in accordance with the manufacturer's protocol (*Roche Diagnostics*, DE-Mannheim; cell proliferation kit II (XTT), cat. No. 11 465 015 001). In brief, 10 000 cells/well of CCRF-CEM and MRC-5 cells were seeded into 96-well plates (100 μl, flat bottom) and treated with various concentrations of **2**–**4** for 72 h. MRC-5 Cells were grown overnight before the test compounds were added. CCRF-CEM Cells were treated immediately. After

72 h, a freshly prepared XTT soln. (5 ml of XTT soln., plus 100 µl of electron coupling reagent) was added and analyzed after another 1.5 (MRC-5 cells) or 4 h (CCRF-CEM cells) using a Victor2 1420 multilabel counter (*PerkinElmer Life Sciences*, Waltham, MA, USA). Vinblastine served as positive control (0.01 µM). The IC_{50} values were determined using SigmaPlot 12.0 (*Systat Software Inc.*, Chicago, IL, USA) and the four-parameter logistic curve. At least five different concentrations and two different cell passages each tested in three independent wells were used.

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