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Antifungal Terpenoids from *Hyalis argentea* var. *latisquama*

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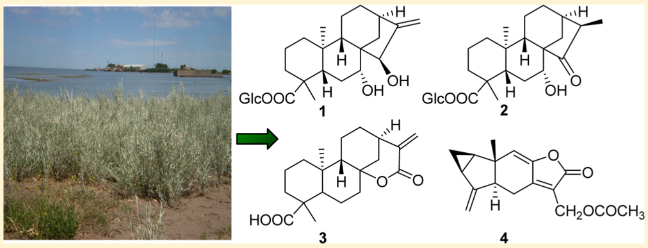
Supporting Information

ABSTRACT: A detailed chemical study of the aerial parts and rhizomes of *Hyalis argentea* var. *latisquama* yielded a variety of sesqui- and diterpenes. In total, 26 compounds were isolated and identified, of which four are new, namely, two *ent*-kaurenes (**1** and **2**), a diterpene lactone (**3**), and a lindenanolide (**4**). The previously reported compounds included a series of lindenanolides, guaianolides, elemanolides, and additional diterpenes. The antifungal activity of the isolated compounds was tested against *Cryptococcus neoformans* and *Candida albicans*. Among the isolated compounds, the lindenanolides were the only structural class that showed strong antifungal activity, and onoseriolide acetate (**5**) was the most active. On the other hand, the isolated guaianolides were only moderately active, while the diterpenes did not show significant antifungal activity.



Hyalis argentea D. Don ex Hook. & Arn. is a sturdy shrub that belongs to the plant family Asteraceae. The genus *Hyalis* is native to South America and is widely distributed in Argentina. In particular, *H. argentea* has three varieties: *argentea*, *macrosperma*, and *latisquama*.[1] Among these, only *H. argentea* D. Don ex Hook. & Arn. var. *latisquama* Cabrera is found along the coastal dunes of the province of Buenos Aires.[2] Previous work on the aerial parts of this species, collected at the arid northwestern province of Catamarca, led to the report of the isolation of a new *ent*-kaurene glycoside and three diterpene lactones in addition to other known compounds and is the only study to date on the chemistry of *Hyalis*.[3] However, in the above-mentioned work, the taxonomic variety was not identified. As part of a systematic survey of the Argentinean natural resources in the search for new antifungal agents, *Hyalis argentea* var. *latisquama* was collected at Puerto Rosales (the east central coastal dunes of the province of Buenos Aires). This is a completely different ecosystem compared to the province of Catamarca, where this species was previously collected. Taking into consideration that the production of secondary metabolites can be drastically modified by edaphoclimatic conditions, a different profile of secondary metabolites was expected.

The ethanolic extracts of the aerial parts and rhizomes of *H. argentea* showed antifungal activity and were studied separately due to their different chromatographic profiles. In total, 26 compounds were isolated and fully characterized. Four of these compounds (**1**−**4**) were new, and another 14 have not been previously reported for this species, while the remaining eight compounds had been previously isolated from this plant.[3] The antifungal activity of *H. argentea* extracts and the isolated compounds was studied against the clinically important fungi *Cryptococcus neoformans* and *Candida albicans*.

RESULTS AND DISCUSSION

The crude ethanolic extracts of the aerial parts and rhizomes of *H. argentea* were partitioned separately (see Experimental Section) to obtain lipophilic and polar extracts. Fractionation of these extracts afforded four new compounds: two *ent*-kaurenes (**1** and **2**), a diterpene lactone (**3**), and an oxyonoseriolide (**4**).

The molecular formula of compound **1** was established as $C_{26}H_{40}O_9$ by HRESIMS ($[M + Na]^+$ m/z 519.2569, calcd 519.2565), which indicated seven degrees of unsaturation. A loss of 162 mass units from the molecular ion suggested the presence of a sugar moiety and a possible structure of a glycosylated diterpene for compound **1**. This assumption was confirmed by the DEPT-HSQC experiment that showed an anomeric carbon at δ_C 95.8, δ_H 5.37 (d, J = 7.8 Hz). Other structural features readily identified by 2D NMR methods were an exomethylene and two hydroxylated methines belonging to the diterpene core (δ_C 159.2, 105.3, 76.0, and 73.0, respectively). Taking into account these NMR data (see Table 1) and by comparison with the previously reported structures of diterpenoids from *H. argentea*,[3] compound **1** was clearly characterized as a dihydroxylated *ent*-kaurene glycoside with an exomethylene replacing a methyl group. The position of this double bond was established as $\Delta^{16,17}$ based on the HMBC correlations of the protons at δ_H 5.06 and 4.94 with δ_C

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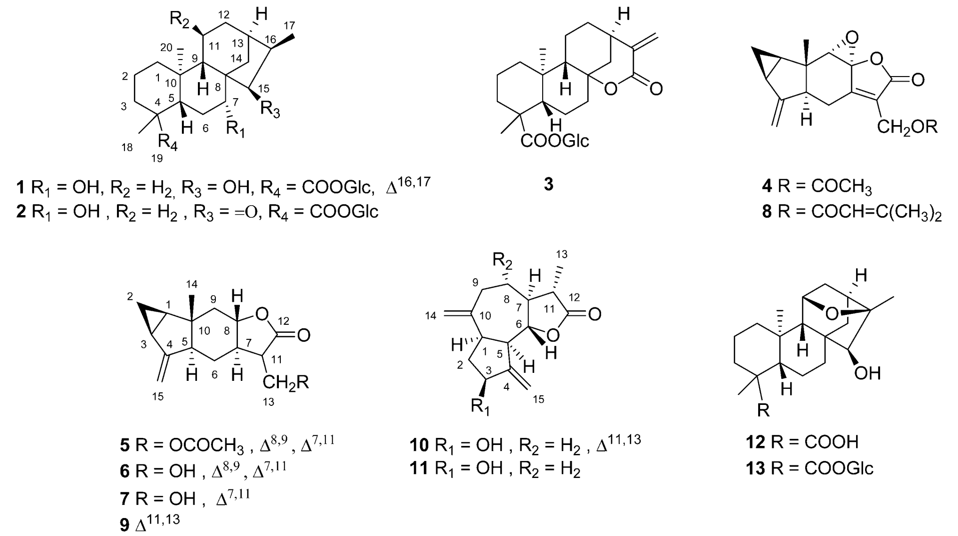
Chart 1



1 R_1 = OH, R_2 = H_2, R_3 = OH, R_4 = COOGlc, $\Delta^{16,17}$
2 R_1 = OH , R_2 = H_2 , R_3 = =O, R_4 = COOGlc

3

4 R = $COCH_3$
8 R = $COCH=C(CH_3)_2$

5 R = $OCOCH_3$, $\Delta^{8,9}$, $\Delta^{7,11}$
6 R = OH , $\Delta^{8,9}$, $\Delta^{7,11}$
7 R = OH , $\Delta^{7,11}$
9 $\Delta^{11,13}$

10 R_1 = OH , R_2 = H_2 , $\Delta^{11,13}$
11 R_1 = OH , R_2 = H_2

12 R = COOH
13 R = COOGlc

41.3 (C-13) and 76.0 (C-15). Additional HMBC correlations [δ_H 3.42 (H-7) with δ_C 55.0 (C-5) and 31.6 (C-6) and δ_H 4.42 (H-15) with δ_C 73.0 (C-7)] placed the remaining hydroxy group at C-7. Finally, an HMBC correlation between the anomeric proton and a carbonyl group at δ_C 178.1 (C-19) indicated that the sugar moiety formed a glycosyl ester. The carbohydrate unit was identified as D-(+)-glucose based on GC analysis of the acetylated alditol derivative obtained after acid hydrolysis of **1** and measurement of its specific rotation. The large coupling constant of the anomeric proton defined a *β*-D-glucoside. The relative configuration at C-7 and C-15 was assigned by interpretation of the coupling constants and NOESY correlations between δ_H 3.42 (H-7) and 1.15 (H-5) and δ_H 4.42 (H-15), 3.42 (H-7), and 1.51 (H-14a). In this way, compound **1** was identified as *ent*-7*α*,15*β*-dihydroxykaur-16-ene-19-*β*-D-glucopyranosyl ester, a previously unreported compound. The NMR data of compound **1** are shown in Table 1.

The HRESIMS of compound **2** was consistent with a molecular formula of $C_{26}H_{40}O_9$ ([M + Na]$^+$ *m/z* 519.2569, calcd 519.2564). A careful examination of the NMR spectra (Table 1) revealed that compound **2** is also a C-19 glycosylated *ent*-kaurene derivative. An analysis of the sugar moiety in the same way as with compound **1** also revealed a *β*-D-glucopyranosyl unit. The ^{13}C NMR and DEPT-HSQC spectra showed the presence of a ketone at C-15 (δ_C 226.0) and a hydroxy group at C-7 (δ_C 72.5). The location of the ketone carbonyl was established on the basis of HMBC correlations between δ_C 226.0 (C-15) and δ 1.08 (H_3-17), 2.14 (H-14a), and 2.26 (H-16). On the other hand, the COSY experiment allowed the connectivity pattern to be established between δ 1.21 (H-5), 2.04 (H_2-6), and 3.74 (H-7). The relative configuration at C-7 was obtained by interpretation of the coupling constants and the NOESY correlations between δ 3.74 (H-7) and δ 0.97 (H-9), 1.21 (H-5), and 2.04 (H_2-6). In this way, compound **2** was identified as the previously unreported *ent*-7*α*-hydroxy-15-oxokauran-19-*β*-D-glucopyranosyl ester.

Compound **3** gave a molecular formula of $C_{26}H_{38}O_9$ as determined by HRESIMS ([M + Na]$^+$ *m/z* 517.2426, calcd 517.2408), suggesting the structure of another glycosylated diterpene. By comparison with the previously reported compounds for this species,[3] compound **3** showed a close resemblance to hyalic acid, but in this case as a *β*-D-glucopyranosyl ester. An HMBC correlation between the C-19 carbonyl and the anomeric proton confirmed that compound **3** is the *β*-D-glucopyranosyl ester of hyalic acid, which is also a new compound. The NMR data of compound **3** are shown in Table 1.

The molecular formula of compound **4** was determined as $C_{17}H_{18}O_5$ ([M + H]$^+$ *m/z* 303.1224, calcd 303.1227), which indicated nine degrees of unsaturation. In the NMR spectra of **4** (Table 2), a methyl group at δ_H 2.10 and a carbonyl at δ_C 170.3, which showed an HMBC correlation, clearly established the presence of an acetate and the possible structure of an acetylated sesquiterpene. The ^{13}C and DEPT-HSQC spectra indicated the occurrence of two double bonds, of which one was an exomethylene (δ_C 106.9, δ_H 5.05, 4.71), and of an additional ester carbonyl at δ_C 169.1 and a single skeletal methyl group (δ_C 17.0, δ_H 0.66). Taking into account the number of unsaturations, four rings had to be present. A characteristic methylene group (δ_C 16.6, δ_H 0.93, 0.86) suggested that compound **4** most likely possesses a cyclopropane ring. An oxidized methylene signal at δ_C 55.4 (δ_H 4.87, 4.82) showed HMBC correlations with the acetate, while the remaining carbonyl was identified as a lactone due to its HMBC correlation with an oxidized quaternary carbon at δ_C 88.0. An additional oxidized methine was also observable (δ_C 64.6, δ_H 4.23). HMBC correlations showed that these two oxidized carbons are vicinal and probably formed an epoxide. A careful analysis of the complete set of 2D NMR spectra led to a lindenanolide skeleton, closely related to onoseriolide, with an acetoxymethylene at C-13 and an epoxide between C-8 and C-9. Thus, compound **4** was identified as the previously unreported 8*α*,9*α*-epoxyonoseriolide acetate.

In addition to compounds **1**−**4**, an exhaustive study of the separate extracts from the rhizomes and aerial parts yielded 22 known compounds, among them a group of five additional lindenanolides (**5**−**9**), of which some displayed antifungal activity. Other isolated compounds included a group of eight guaianolides, of which two (**10** and **11**) were also weakly antifungal, two elemanolides, a diterpene lactone, umbelliferone, and five *ent*-kaurenes. Two of these *ent*-kaurenes (**12** and **13**) were previously regarded as extraction artifacts. However, in the present study, additional evidence was found to support their natural origin. A list of all the compounds isolated from *H.*

Table 1. NMR Spectroscopic Data (500 MHz, CD_3OD) for Compounds **1**−**3**

	1			**2**			**3**		
position	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC
1	41.6, CH_2	1.92, m		40.7, CH_2	1.82, d (13.3)		42.2, CH_2	1.95, m	2
		0.86, m			0.78, td (13.3, 12.5, 4.2)			1.03, m	
2	20.0, CH_2	1.92, m		19.7, CH_2	1.95, m		20.2, CH_2	1.92, m	4, 10
		1.45, m			1.43, m			1.47, m	
3	38.9, CH_2	2.20, m	19	38.8, CH_2	2.18, dt (13.4, 3.2)		38.7, CH_2	2.22, d (13.4)	1, 2, 4, 5, 19
		1.08, m			1.08, m			1.11, dd (11.4, 2.6)	
4	44.8, C			44.9, C			44.9, C		
5	55.0, CH	1.15, m	1, 3, 4, 6, 7, 10, 19	55.0, CH	1.21, m	10	57.7, CH	1.30, dd (11.4, 2.6)	4, 6, 10, 18, 20
6	31.5, CH_2	2.06, m	4, 5, 7, 8, 10	30.8, CH_2	2.04, m	5, 7, 8, 10	21.8, CH_2	1.97, m	
7	73.0, CH	3.42, m	5, 6	72.5, CH	3.74, m		43.6, CH_2	1.96, m	
								1.75, m	
8	52.4, C		6, 9, 13, 14	59.7, C			87.3, C		
9	46.1, CH	1.33, d (6.8)	8, 10, 11, 12, 14, 20	52.7, CH	0.97, m	10	52.8, CH	1.52, m	8, 10, 12, 14, 20
10	40.5, C			40.6, C			41.3, C		
11	18.9, CH_2	1.67, m	9	19.0, CH_2	1.65, m		17.6, CH_2	1.81, m	8, 9, 10, 13
		1.50, m			1.16, m			1.53, m	
12	34.9, CH_2	1.67, m	9, 14	26.2, CH_2	1.70, m		31.7, CH_2	1.96, m	
		1.46, m						1.51, m	
13	41.3, CH	2.62, brs	12	35.8, CH	2.45, m		35.6, CH	2.97, m	15
14	28.8, CH_2	1.61, d (12.0)	8, 9, 12, 13, 15	29.8, CH_2	2.14, m	15	34.7, CH_2	2.45, dd (14.2, 3.3)	13, 16
		1.51, m			1.94, m			1.70, m	
15	76.0, CH	4.42, brt	7, 8, 9, 17	226.0, C			168.7, C		
16	159.2, C			49.7, CH	2.26, t (6.8)	13, 15, 17	141.3, C		
17	105.3, CH_2	5.06, brt	13, 15	10.2, CH_3	1.08, d (6.8)	15	127.8, CH_2	6.34, d (1.6)	14, 15, 16
		4.94, d (2.6)						5.64, dd (1.6, 0.6)	
18	28.8, CH_3	1.25, s	2, 3, 4, 5, 19	28.8, CH_3	1.25, s	3, 4, 5, 19	28.9, CH_3	1.26, s	3, 4, 5, 19
19	178.1, C			177.8, C			178.1, C		
20	16.4, CH_3	0.98, s	5, 9, 10	16.1, CH_3	1.03, s	5, 9, 10	17.0, CH_3	0.99, s	5, 9, 10
1′	95.8, CH	5.37, d (7.8)	19, 5′	95.8, CH	5.38, d (7.7)	19	95.8, CH	5.40, d (7.9)	19
2′	74.0, CH	3.37, m		74.0, CH	3.38, m		74.0, CH	3.35, m	
3′	78.7,[a] CH	3.36, m		78.7,[a] CH	3.37, m		78.8,[a] CH	3.37, m	
4′	71.1, CH	3.36, m		71.1, CH	3.36, m		71.0, CH	3.37, m	
5′	78.5,[a] CH	3.36, m		78.6,[a] CH	3.41, m		78.7,[a] CH	3.41, m	
6′	62.4, CH_2	3.81, dd (12.1, 1.6)		62.4, CH_2	3.83, dd (12.1, 1.5)		62.3, CH_2	3.83, dd (12.3, 1.6)	
		3.69, dd (12.5, 3.8)			3.69, dd (12.1, 4.1)			3.69, m	

[a] May be interchanged.

argentea var. *latisquama*, arranged by structural class, is provided in the Supporting Information.

Lindenanolides are a class of compounds with limited occurrence and have not been previously reported from the genus *Hyalis*. Besides the new compound 8α,9α-epoxyonoseriolide acetate (**4**), onoseriolide acetate (**5**) has not been previously isolated as a natural product, but was reported as a semisynthetic compound obtained from onoseriolide (**6**).[4] The remaining lindenanolides were identified as 8α,9α-epoxyonoseriolide senecioate (**7**),[5] 13-desoxyisoonoseriolide (**8**), and 8β,9-dihydro-onoseriolide (**9**).[6] Other isolated sesquiterpene lactones in this work were the elemanolide igalan and its diasteromer[7] and eight guaianolides: dehydrocostus lactone, mokko lactone,[8] zaluzanin C (**10**),[9] 11,13-dihydrozaluzanin C (**11**),[9,10] 11,13-dihydrodesacylcynaropicrin,[11] macrocliniside F,[12] glucozaluzanin C,[13] and 11,13α-dihydroglucozaluzanin C.[14]

In addition to the new diterpenes **1**−**3**, members of a group of known compounds sharing the same skeleton were also isolated: *ent*-11α-hydroxy-15-oxo-kaur-16-en-19-oic acid,[15] paniculoside II,[16] hyalic acid, and (7*R*)-*ent*-15-oxokaur-16-en-7-ol-19-oic acid β-glucopyranosyl ester.[3] The last two compounds were previously isolated from *H. argentea*. Finally, umbelliferone was also isolated.[3]

Two additional *ent*-kaurenes with an oxo bridge between C-11 and C-16 (**12** and **13**) were also isolated in this work. There was a previous controversy about the origin of these substances, but their isolation in the present work may clarify their natural origin. Compound **12** was previously isolated from *Eupatorium album*,[15] together with the aglycone of paniculoside II. In this study, the authors concluded that **12** is an artifact formed from

Table 2. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for Compound 4

position	δ_C, type	δ_H (J in Hz)	HMBC	COSY
1	23.7, CH	1.72, m		2, 3
2	16.6, CH_2	0.93, dd (14.4, 7.2)		
		0.86, dd (13.1, 9.0)		
3	22.9, CH	2.01, m		1, 2
4	149.6, C			
5	50.2, CH	3.40, brd		6, 15
6	21.6, CH_2	2.74, dd (19.9, 5.6)	5, 7, 8, 10, 11	5
		2.33, dd (19.9, 13.5)		
7	158.2, C			
8	88.0, C			
9	64.6, CH	4.23, brs	1, 5, 8, 10	14
10	41.2, C			
11	127.2, C			
12	169.1, C			
13	55.4, CH_2	4.82, d (13.4)	170.3, 7, 11, 12	6
		4.87, d (13.4)		
14	17.0, CH_3	0.66, s	1, 5, 9, 10	
15	106.9, CH_2	5.05, d (1.7)	1, 5	
		4.71, m		
$COCH_3$	170.3, C			
	20.5, CH_3	2.10, s	170.3	

the latter compound by traces of acid present in the $CDCl_3$ used for NMR spectra. However, in a later investigation, compound **12** was isolated from *Nouelia insignis*,[17] this time without the presence of the possible precursor. In the present study, both compounds **12** and **13** (the glycosylated form of **12**) were isolated, as well as paniculoside II. Compound **13** was also previously isolated from *Stevia paniculata*[18] from a fraction rich in paniculoside II, and the authors stated that **13** was probably an artifact, since they worked with solvent mixtures containing chloroform. However, in the present work, chloroform was never used in any of the purification steps, and the NMR spectra were recorded in CD_3OD, but compound **13** was still isolated herein. Furthermore, the aglycone of paniculoside II, the possible precursor of compound **12**, was not detected in the extract. These data clearly suggest that compounds **12** and **13**, with an oxo bridge between C-11 and C-16, may be of natural origin.

Considering the previous reports of antifungal activity of compounds closely related to those isolated in the present work,[19−21] the crude ethanolic extracts of the rhizomes and aerial parts as well as the isolated compounds were tested against *C. albicans* and *C. neoformans* in microdilution assays, which followed the guidelines of the Clinical and Laboratory Standards Institute (CLSI).[22] The fungal strains were selected based on the clinical relevance associated with their related infections. *C. albicans* accounts for more than 60% of all isolates from clinical fungal infections,[23] and in the last 10 years there has been a surge of resistant strains,[24] thereby promoting a renewed search for compounds with anti-*C. albicans* activity.[25] On the other hand, *C. neoformans* produces cryptococcal meningitis, which has killed more than 650 000 immunocompromised patients worldwide. Amazingly, the treatment for this infection is still based on drugs discovered nearly 50 years ago.[26]

The percentages of fungal growth inhibition produced by the ethanolic extracts of the aerial parts and rhizomes of *H. argentea* var. *latisquama* at different concentrations were calculated with the aid of a microplate reader (see Experimental Section), and the results are shown in Figure S27 (Supporting Information). Both ethanolic extracts of rhizomes and aerial parts from *H. argentea* var. *latisquama* displayed antifungal activity at the different test concentrations used. However, it is clear that the rhizome EtOH extract showed higher percentage inhibitions against both fungi at concentrations lower than 250 μg/mL.

The antifungal activity of the isolated compounds against *C. albicans* or *C. neoformans* was assayed by bioautography TLC plates (see Experimental Section).[27,28] This bioassay showed that only lindenanolides **4**−**6** and 11,13-dihydrozaluzanin C (**11**) were active against both fungi, while zaluzanin C (**10**) displayed activity only against *C. albicans*. Guided by these results, all active compounds were tested in microdilution assays.[22] Lindenanolides **4**−**6** were tested against *C. albicans* ATCC 10231 and *C. neoformans* ATCC 32264, and their inhibition percentages at different concentrations were plotted as shown in Figure 1.

These results showed that compound **5** was the most active among the lindenanolides, exhibiting 50, 80, and 100% inhibition at the following concentrations: 15.6, 62.5, and 125 μg/mL (Figure 1A) against *C. albicans* and 15.6, 31.25, and 62.5 μg/mL against *C. neoformans* (Figure 1B). Compound **6**, which is the C-13 deacetylated form of **5**, displayed a lower activity (50% inhibition at concentrations ≥ 125 μg/mL). In turn, compound **4**, which differs from **5** in the epoxidation of $\Delta^{8,9}$, showed very low inhibition percentages even at 250 μg/

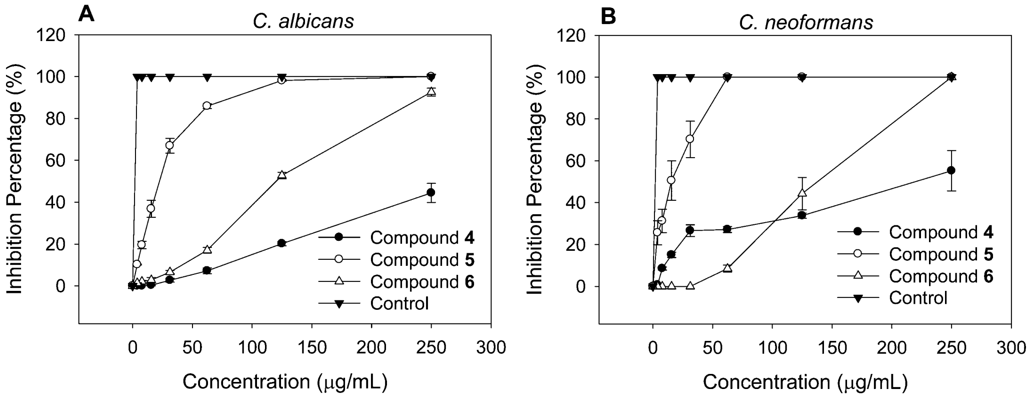


Figure 1. Fungal growth inhibition percentages of (A) *C. albicans* and (B) *C. neoformans* by lindenanes **4**−**6** at different concentrations. Control: Amphotericin B.

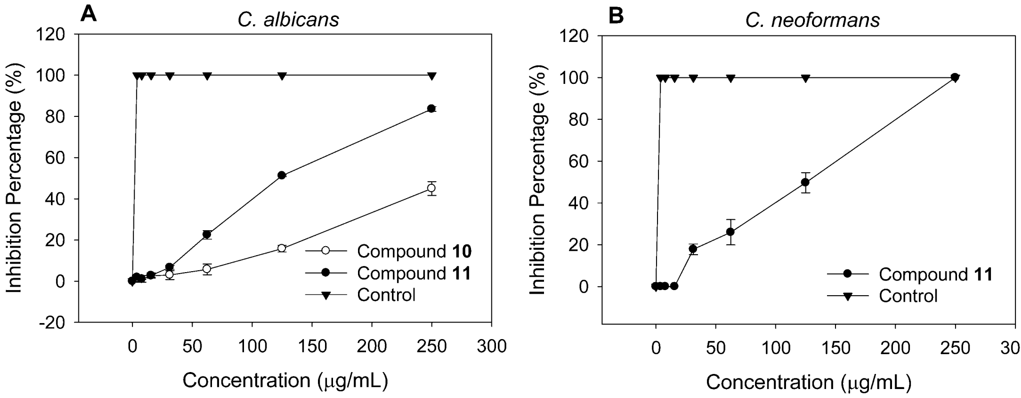


Figure 2. Fungal growth inhibition percentages of (A) *C. albicans* and (B) *C. neoformans* by guaianolides **10** and **11** at different concentrations. Control: Amphotericin B.

mL, while compound 7, which lacks the $\Delta^{8,9}$ double bond, was inactive. These results show that the $\Delta^{8,9}$ double bond may play a significant role in the antifungal activity.

The results of the antifungal activity assays of the guaianolides zaluzanin C (**10**) and 11,13-dihydrozaluzanin C (**11**) are shown in Figure 2. Compound **10** showed the best antifungal activity against *C. albicans* (Figure 2A) and was the only guaianolide that exhibited a moderate activity against *C. neoformans* (MIC_{50} = 125 μg/mL, Figure 2B). Both active guaianolides share the presence of a β-OH group at C-3. Glycosylation at this position produces a complete loss of activity, as well as an additional hydroxy group at C-8. These results show that a 3β-OH appears to be necessary, but not sufficient, for the antifungal activity in guaianolides. The higher activity of 11,13-dihydrozaluzanin C (**11**) versus zaluzanin C (**10**) may be explained by the absence of the double bond between C-11 and C-13. None of the isolated diterpenes were active in the microdilution assays used.

The small amount of compound **5** (0.9 mg) isolated prevented further tests such as fungicide capacity, time-kill assays, new tests in clinical isolates, or studies on the mechanism of action. However, the present results show that the lindenane skeleton may be a scaffold for the development of new antifungal compounds.[25,29]

This work showed that the rhizomes and aerial parts of *H. argentea* var. *latisquama* are a rich source of terpenoids with different scaffolds, since 26 compounds comprising six lindenanolides, two elemanolides, eight guaianolides, nine *ent*-kaurenes, and a coumarin were isolated and characterized. Four of these, the *ent*-kaurenes **1**−**3** and the lindenanolide **4**, are new compounds. This is the first report of lindenanolides in the genus, as well as of the antifungal activity of this class of sesquiterpene lactones. A different distribution of secondary metabolites was detected in both plant parts studied: lindenanolides and glycosylated *ent*-kaurenes were found mainly in the rhizomes, while guaianolides were mostly isolated from the aerial parts. Taking into consideration that the observed profile of secondary metabolites was highly different compared to that obtained in the previous study,[3] in which lindenanolides were not reported, it will be interesting to study the regional variability of these antifungal compounds of *H. argentea*.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. UV spectra were obtained on a Hewlett-Packard 8453 spectrophotometer, and IR spectra were obtained on an FT-IR Nicolet Magna 550 instrument. ^{1}H and ^{13}C NMR data were measured in Bruker Avance-2 (500 MHz) and AC-200 (200 MHz) spectrometers, in $CDCl_3$ or CD_3OD. Proton chemical shifts were referenced to the residual signal of $CHCl_3$ at δ 7.26 or 3.31 when CD_3OD was used, and ^{13}C NMR were referenced to the central peak of $CDCl_3$ at 77.0 ppm or CD_3OD at 49.0 ppm. Homonuclear ^{1}H connectivities were determined by COSY experiments. The edited reverse-detected single quantum heteronuclear correlation (DEPT-HSQC) experiment allowed the determination of carbon multiplicities, as well as one-bond proton−carbon connectivities, and the heteronuclear multiple bond correlation (HMBC) experiment allowed the determination of long-range proton−carbon connectivities. The relative configuration was determined by gradient-enhanced NOESY experiments. All 2D NMR experiments were performed using standard pulse sequences. HRESI mass spectra were recorded using a MicrOTOF QII Bruker mass spectrometer. Reversed-phase vacuum flash chromatography was carried out on octadecyl-functionalized silica gel (Aldrich Chemical Co.). HPLC separations were performed using HPLC-grade solvents, a Thermo Separations Spectra Series P100 pump, a Thermo Separations Refractomonitor IV RI detector, a Thermo Separations SpectraSeries UV 100 UV detector, and a YMC RP-18 (5 μm, 20 mm × 250 mm) column. UV detection was performed at 220 nm. Analytical HPLC was carried out on a Gilson 506C instrument, equipped with a 322 pump and a UV detector (Gilson 170). A Phenomenex Synergi Fusion-RP (4 μm, 4.60 mm × 150 mm) column was used with a 0.2 mL/min flow rate. Gradient elution was performed using (A) MeOH and (B) H_2O, starting with 70% A and raising to 100% MeOH within 20 min, followed by 5 min in isocratic mode. Sephadex LH-20 was obtained from Pharmacia Inc., and TLC was carried out on Merck silica gel 60 F_{254} plates. TLC plates were sprayed with 2% vanillin in concentrated H_2SO_4. All other solvents were distilled prior to use. The purity of the compounds tested was assessed by ^{1}H NMR (500 MHz) and HPLC. All active compounds showed purities higher than 95% by HPLC analysis.

Plant Material. Specimens of *H. argentea* var. *latisquama* were collected at Puerto Rosales (lat. 38°54′39.6″ S and long. 62°4′17.1″ W), Partido de Coronel Rosales, Province of Buenos Aires, Argentina, in December 2009 (summer). Authentication of the plant material was carried out by Dr. S. E. Freire at the Darwinion Institute, San Isidro, Buenos Aires, Argentina. A voucher specimen (SI 165956, bar code: 046515) was deposited at the Darwinion Institute.

Extraction and Isolation. Fresh aerial parts (2480 g) and rhizomes (373 g) were separated and ground before maceration in EtOH at room temperature for 5 days. After filtration, the solvent was evaporated under reduced pressure to yield syrupy residues (180 and 14 g, respectively). Each residue was partitioned between MeOH−H_2O (9:1) and cyclohexane to obtain, after evaporation at reduced pressure, the corresponding polar and lipophilic subextracts. The yields obtained for the different extracts were 10 g of polar rhizomal extract (ROP), 3.9 g of lipophilic rizhomal extract (ROL), and 30 g of polar aerial parts (AOP). Chromatographic and ^{1}H NMR spectroscopic analysis of the lipophilic extract of the aerial parts (AOL) showed that it consisted mainly of fats, and for this reason it was not further investigated.

An aliquot of the rhizome polar phase (240 mg) was permeated through a Sephadex LH-20 column (30 cm) using MeOH as eluent. Fractions S8−S11 were subjected to dry column flash chromatography on silica gel with an CH_2Cl_2−EtOAc gradient, affording compounds **5** (0.9 mg), **6** (1.6 mg), **7** (0.9 mg), and 11,13-dihydrodesacylcynaropicrin (1.4 mg), and an impure fraction, ROP7 (35 mg). The latter was further purified by HPLC eluting with MeOH−H_2O (7:3) to obtain compounds **1** (8 mg), **2** (1.1 mg), **3** (14.1 mg), hyalic acid (1.7 mg), and (7*R*)-*ent*-15-oxokaur-16-en-7-ol-oic acid *β*-glucopyranosyl ester (2.6 mg).

The lipophilic extract of the rhizomes was subjected to dry column flash chromatography on silica gel (cyclohexane−CH_2Cl_2 gradient), affording four fractions (ROL1 to ROL4). Igalan (6.1 mg), its diastereoisomer (1.9 mg), dehydrocostus lactone (3.3 mg), and 13-desoxyisoonoseriolide (1.6 mg) were obtained from ROL1 (136.0 mg, eluted with cyclohexane−CH_2Cl_2, 1:1) after HPLC purification with MeOH−H_2O (75:25). Purification of ROL2 (81 mg, eluted with cyclohexane−CH_2Cl_2, 3:7) and ROL3 (50 mg, eluted with cyclohexane−CH_2Cl_2, 4:6) by preparative HPLC under the same conditions afforded compounds **8** (0.9 mg) and mokko lactone (2.3 mg), respectively. In addition, HPLC separation of ROL4 (80 mg, eluted with MeOH−H_2O, 7:3) yielded an impure fraction (7.6 mg), which was further purified by preparative TLC (CH_2Cl_2 as mobile phase) to obtain compound **4** (3.3 mg).

The polar phase from the aerial parts extract was subjected to dry column flash chromatography on silica gel (CH_2Cl_2−EtOAc−MeOH gradient), yielding seven fractions (AOP1−AOP7). The same chromatographic procedure was applied to AOP1 (1.335 g, eluted with CH_2Cl_2−EtOAc, 1:1) under isocratic conditions (CH_2Cl_2−EtOAc, 9:1). After TLC comparison, fractions showing similar TLC patterns were pooled into two groups, AOP1.1 (280.5 mg) and AOP1.2 (478.6 mg).

Umbelliferone (93 mg) was obtained from the MeOH-insoluble portion of AOP1.1. The MeOH-soluble portion was purified by preparative HPLC with MeOH−H_2O (6:4) as eluent to obtain the pure compounds **6** (3 mg), **10** (3.3 mg), **11** (3.2 mg), **12** (19 mg), and umbelliferone (16 mg). AOP1.2 (150 mg) was subjected to HPLC under the same conditions to yield 11,13-dihydrodesacylcynaropicrin (6.6 mg), **12** (29 mg), and *ent*-11*α*-hydroxy-15-oxo-kaur-16-en-19-oic acid (31.8 mg).

AOP6 (eluted with EtOAc−MeOH, 3:7, 452.5 mg) was permeated through a Sephadex LH-20 column (30 cm) using cyclohexane−CH_2Cl_2−MeOH (2:2:1) as mobile phase. After TLC comparison, fractions showing similar TLC patterns were pooled into two groups, AOP6.1 (135.4 mg) and AOP6.2 (266.6 mg). HPLC purification of AOP6.1 with CH_3CN−H_2O (2:8) afforded glucozaluzanin C (5.4 mg), 11,13*α*-dihydroglucozaluzanin C (7.5 mg), and **13** (4.0 mg). Paniculoside II (3.4 mg) and macrocliniside F (40.8 mg) were obtained from AOP6.2 by applying these same purification conditions.

ent-7α,l5β-Dihydroxykaur-l6-ene-19-β-D-glucopyranosyl ester ***(1):*** amorphous solid; $[\alpha]^{25}_{D}$ −159.2 (*c* 0.050, MeOH); UV (MeOH) λ_{max} (log *ε*) 214 (4.20), 275 (3.29) nm; IR (KBr) ν_{max} 3386, 2931, 1726, 1661 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* ([M + Na]$^+$ 519.2569, calcd for $C_{26}H_{40}O_9Na$ 519.2565).

ent-7α-Hydroxy-l5-oxokauran-19-β-D-glucopyranosyl ester (2): amorphous solid; $[\alpha]^{25}_{D}$ −172.8 (*c* 0.045, MeOH); UV (MeOH) λ_{max} (log *ε*) 214 (4.03) nm; IR (KBr) ν_{max} 3355, 2929, 2867, 1725 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* ([M + Na]$^+$ 519.2569, calcd for $C_{26}H_{40}O_9Na$ 519.2564).

Hyalic acid β-D-glucopyranosyl ester (3): amorphous solid; $[\alpha]^{25}_{D}$ −157.3 (*c* 0.050, MeOH); UV (MeOH) λ_{max} (log *ε*) 215 (4.17) nm; IR (KBr) ν_{max} 3441, 2958, 2926, 2864, 1722, 1625 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* ([M + Na]$^+$ 517.2426, calcd for $C_{26}H_{38}O_9Na$ 517.2408).

8α,9α-Epoxyonoseriolide acetate (4): amorphous solid; $[\alpha]^{25}_{D}$ +59.7 (*c* 0.050, MeOH); UV (MeOH) λ_{max} (log *ε*) 249 (3.20) nm; IR (KBr) ν_{max} 2964, 2915, 2845, 1784, 1741 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* ([M + H]$^+$ 303.1224, calcd for $C_{17}H_{19}O_5$ 303.1227).

Glycoside Hydrolyses and Carbohydrate Identification. Hydrolysis of the new glycosides was carried out in 2 M trifluoroacetic acid (90 min, 120 °C). Hydrolysates were derivatized to the alditol acetates (CF_3COOH−Ac_2O, 1:1, 10 min, 50 °C) and analyzed by GLC using a capillary column (30 m × 0.25 mm) coated with SP-2330 (0.20 *μ*m) on a HP-5890 gas chromatograph equipped with a flame ionization detector. Chromatography runs were isothermal at 220 °C, while the injector and detector were set at 240 °C.[30] Standards of acetylated alditols of fucose, arabinose, rhamnose, glucose, and galactose were used. The retention time of the sample in the GC analysis was comparable to that of the glucose standard (t_R: 26.295 min). In order to confirm D or L configuration for glucose, the specific rotation of a 0.70% solution of the hydrolysate in water was measured.

Microorganisms and Media. For the antifungal activity evaluation, standardized strains from the American Type Culture Collection (ATCC, Rockville, MD, USA) were used: *Candida albicans* ATCC 10231 and *Cryptococcus neoformans* ATCC 32264. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid), and subcultured every 15 days to prevent pleomorphic transformations. Inocula of cell suspensions were obtained according to reported procedures and adjusted to (1−5) × 10^3 cells/spores with colony forming units (cfu)/mL.[22]

Bioautography. Warm Sabouraud agar with 0.02% phenol red (1 mL/cm^2) containing a *C. neoformans* (or *C. albicans*) inoculum of (1−5) × 10^5 cells/mL was distributed over previously seeded TLC plates, in triplicate. After solidification of the medium, the TLC plates were put into sterile Petri dishes with covers[27,28] and incubated overnight at 30 °C. Subsequently, the bioautograms were sprayed with an aqueous solution (1 mg/mL) of methyl thiazolyl tetrazolium chloride (MTT) and further incubated for 2 h at 28 °C. Yellow inhibition zones appeared against a dark brown background.

Fungal Growth Inhibition Percentage Determination. Broth microdilution techniques were performed in 96-well microplates according to the guidelines of the Clinical and Laboratory Standards Institute for yeasts (M27-A3).[22] For the assay, compound test wells (CTWs) were prepared with stock solutions of each compound in DMSO (maximum concentration ≤1%), diluted with RPMI-1640, to final concentrations of 250−0.98 *μ*g/mL. An inoculum suspension (100 *μ*L) was added to each well (final volume in the well = 200 *μ*L). A growth control well (GCW) (containing medium, inoculum, and the same amount of DMSO used in a CTW, but compound-free) and a sterility control well (SCW) (sample, medium, and sterile water instead of inoculum) were included for each fungus tested. Microtiter trays were incubated in a moist, dark chamber at 30 °C for 48 h for *C. albicans* or *C. neoformans*. Microplates were read in a VERSA Max microplate reader (Molecular Devices, Sunnyvale, CA, USA). Amphotericin B was used as positive control. Tests were performed in triplicate. Reduction of growth for each compound concentration was calculated as follows: % of inhibition = $100 - (OD_{405}\ CTW - OD_{405}\ SCW)/(OD_{405}\ GCW - OD_{405}\ SCW)$. The means ± SEM were used for constructing the curves representing % inhibition vs concentration of each compound, with SigmaPlot 11.0 software being used.

Statistical Analysis. The unpaired Student's *t* test was used to analyze data ($p < 0.5$, calculated using a two-tailed test, was considered significant).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra of compounds **1**−**4**, structures of all isolated compounds, NMR assignments for compounds **8**, **10**, **12**, igalan's diastereoisomer, mokko lactone, dehydrocostus lactone, macrocliniside F, and hyalic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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