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Antimicrobial activities of sesquiterpene lactones and inositol derivatives from *Hymenoxys robusta*

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ABSTRACT

Six compounds from the aerial parts of the Argentinean plant *Hymenoxys robusta* (Rusby) Parker were isolated and their structures elucidated using extensive spectroscopic analyses. These compounds comprise two inositol derivatives and four 3,4-seco-pseudoguaianolides, including vermeerin. Bioactivity assays of these compounds against bacterial and fungal pathogens showed that only vermeerin possessed antimicrobial activity specific against *Staphylococcus aureus*, and showed no toxicity when exposed to human-derived macrophages.

1. Introduction

The genus *Hymenoxys* belongs to the family Asteraceae, tribe Helenieae, subtribe Tetraneurinae and comprises approximately 62 species widely distributed throughout the world (Baldwin and Wessa, 2000). Previous reports have shown that this genus produces sesquiterpene lactones of the guaianolide, pseudoguaianolide and secopseudoguaianolide types, as well as flavonoids, inositol derivatives and monoterpenes (Herz et al., 1970; Gao et al., 1990; Ahmed et al., 2002). In addition, several *Hymenoxys* species are known as poisons to livestock such as the Western bitterweed (*H. odorata*) (Ivie et al., 1975). Their toxicity is mainly due to the secohelenanolide termed 10α-methylsecopseudoguaianolide hymenoxon, a dihemiacetal sesquiterpene lactone (Ivie et al., 1975; Hill et al., 1977), which have also been demonstrated to possess cytotoxic effects (Ivie et al., 1975).

In this work the species *Hymenoxys robusta* (Rusby) Parker was studied, as which is known in Bolivia as Q'illu Q'illu. It has also been shown to possess poisonous activity on livestock (Zabala and León, 2010); herein, we report isolation and structure elucidation of the new natural secopseudoguaianolides **1–3**, the new inositol derivatives **5–6**, and the known compound vermeerin (**4**). In addition, the bioactivities of these compounds are also reported.

2. Results and discussion

A total of six compounds were isolated from chloroform extract of leaves of *H. robusta* after purification through silica gel column chromatography and reversed-phase HPLC. Three compounds were new natural secopseudoguaianolides (**1–3**), two were new inositol derivatives (**5** and **6**), and the known sesquiterpene dilactone vermeerin (**4**) (Herz et al., 1970).

Compound **1** showed IR bands for hydroxyl and γ-lactone groups at 3409 and 1769 cm^{-1}, respectively. The molecular formula obtained $C_{15}H_{24}O_4$ was followed by HRMS, which showed a $[M+H]^+$ at *m/z* 269.1748 (calcd for $C_{15}H_{25}O_4$ 269.1753), accounting for 4° of unsaturation, and LRMS, which showed a peak at *m/z* 251 (100%) $[M+H-H_2O]^+$, indicating the presence of a hydroxyl group. The ^{1}H-NMR spectrum of **1** (Table 1) showed one methyl singlet at δ 1.05, assigned to CH_3-15, and two methyl doublets at δ 1.07 and 1.14, assigned to CH_3-14 and CH_3-13 respectively. The presence of a saturated γ-lactone ring was corroborated with the double-quartet signal at δ 2.89 (*J* = 9, 7 Hz) assigned to H-11. The hydroxyl group on C4 was evident from the broad singlet at δ 4.55 assigned to H-4, this resonance broadening being due to the W-type long

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Table 1
^{1}H NMR spectroscopic data for compounds **1–3** (200 MHz, $CDCl_3$, TMS standard, δ values).

H	1	2	3
H-1	1.75 dd (11, 5.6; 4)	2.18 m	2.18 m
H-2	1.40–1.66 m	1.40–1.70 m	1.40–1.70 m
H-3α	3.96 ddd (11.8; 11.8; 4.2)	0.99 t (6.6)	1.01 t (8)
H-3β	3.63 ddd (11.8; 4.8; 1.7)	–	–
H-4	4.55 br s	4.70 br d (5.1)	4.42 br d (3.5)
H-6α	1.51 dd (15; 2.2)	2.02 dd (14; 3.3)	2.02 dd (14; 3.3)
H-6β	1.24 dd (15; 11)	1.21 dd (14; 10.3)	1.21 dd (14; 10.3)
H-7	2.97 dddd (11; 9; 5.7; 2.2)	3.10 dddd (11; 10.3; 3.6; 3.3; 3.0)	3.10 dddd (11; 10.3; 3.6; 3.3; 2.9)
H-8	4.71 ddd (8.6; 6.6; 5.7)	3.80 ddd (11; 11; 4.4)	4.00
H-9α	1.90 ddd (15.1; 6.6; 4.3)	2.20 m	2.20 m
H-9β	1.93 ddd (15.1; 10.7; 8.6)	1.50 m	1.50 m
H-10	1.60 m	1.66 m	1.66 m
H-11	2.89 dq (9; 7)	–	–
H-13a	1.14 d (7)	6.15 d (3.6)	6.16 d (3.6)
H-13b		5.42 d (3.0)	5.49 d (2.9)
H-14α	1.07 d (7.4)	3.68 ddd (J = 11.4, 5.1 1.1)	3.88 m
H-14β	–	4.0 dd (J = 11.4, 3.3)	3.48 ddd (J = 11.4; 11.4; 3.5)
H-15	1.05 s	1.02 s	0.98 s

range coupling with H-3β, indicating that the hydroxyl group on C-4 is α-oriented. On the other hand, the signal at δ 4.71 (ddd, J = 8.6; 6.6; 5.7 Hz) were assigned to H-8, while those at δ 3.96 (ddd, J = 11.8; 11.8; 4.2 Hz) and 3.63 (ddd, J = 11.8; 4.8; 1.7 Hz) was assigned to H-3α and H-3β, respectively. The minimum energy conformation of **1** (Fig. 1A) was obtained using the PCMODEL program (Burket and Allinger, 1982), the dihedral angle H-7α-C-7-C-8-H-8α was −25° corresponding to the calculated coupling constant value $J_{7\alpha,8\alpha}$ = 6.4 Hz while the observed value was 5.7 Hz, indicating a *cis* lactone ring closure. On the other hand, the dihedral angles H-7α-C-7-C-11-H-11α, H-10α-C-10-C-1-H-1α, H-10α-C-10-C-9-H-9α, and H-10α-C-10-C-9-H-9β were 30°, 38°, 53°, and 166°, respectively, corresponding to the calculated coupling constant values, $J_{7\alpha,11\alpha}$ = 8.3 Hz, $J_{10\alpha,1\alpha}$ = 6.4 Hz, $J_{10\alpha,9\alpha}$ = 4.3 Hz, and $J_{10\alpha,9\beta}$ = 11.8 Hz, while the observed values for these coupling constants were 9.0, 5.6, 4.3, and 10.7 Hz, respectively. All these facts indicate that both CH_3-13 and CH_3-14 are β-oriented. Observed and calculated coupling constant values are in good agreement;

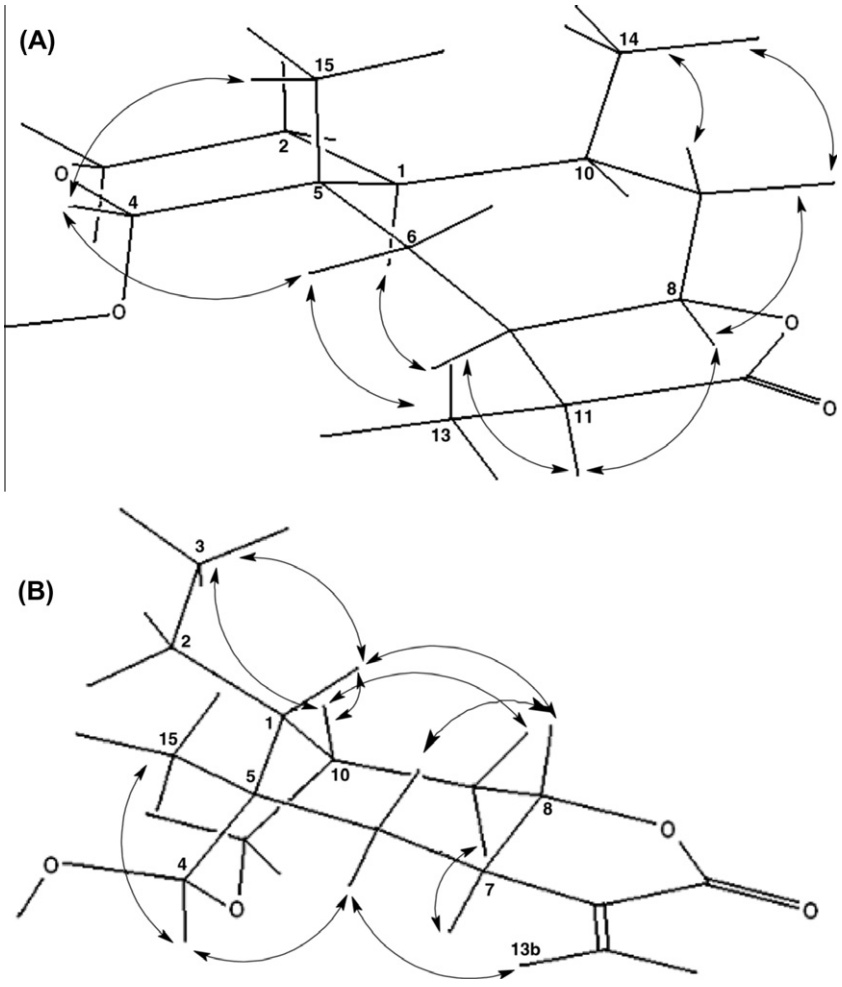

Fig. 1. (A) Minimum energy conformation of 1 and NOESY correlations. (B) Minimum energy conformation of 2 and NOESY correlations.

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dihedral angles, along with calculated and observed coupling constants, are listed in Table 3. NOESY experiments were performed to confirm the relative configuration described above, especially at the lactone ring closure. Correlations were found between H-8 and H-9α and H-11, as well as between H-7 and H-1 and H-11. These results support the stereochemistry shown in structure **1** (more correlations are depicted in Fig. 1A). On the other hand, ^{13}C-NMR data (Table 2) showed 15 carbons and the DEPT experiments indicated that the protonated carbons belong to three methyls, four methylenes, one of which was oxygenated, six methines, two of which were oxygenated. All assignments were achieved with the aid of gCOSY, gHSQC, gHMBC, and DEPT experiments. Although a previous work reported the synthesis of a similar compound from hymenoxynin (Herz et al., 1970), its stereochemistry was not specified.

Compounds **2** and **3** were obtained as a 3:1 mixture. The IR spectrum of the mixture showed the presence of a α,β-unsaturated γ-lactone (1746, 1650 cm^{-1}), and a hydroxyl group (3409 cm^{-1}). HRMS gave only one $[M+H]^+$ at *m/z* 267.1606 (calcd for $C_{15}H_{23}O_4$ 267.1596) indicating a molecular formula of $C_{15}H_{22}O_4$, which accounted for 5° of unsaturation. The ^{1}H-NMR spectrum of the major compound showed characteristic doublets of a *trans*-γ-lactone ring at δ 6.15 (*J* = 3.6 Hz) and δ 5.42 (*J* = 3.0 Hz) corresponding to H-13a and H-13b, respectively. On the other hand, the broad doublet at δ 4.70 (*J* = 5.1 Hz) was assigned to H-4, the signals at δ 4.00 (dd, *J* = 11.4 and 3.3 Hz) and 3.68 (ddd, *J* = 11.4, 5.1 and 1.1 Hz) were assigned to H-14β and H-14α, respectively; the multiplicity of H-14α indicates a W-type coupling with H-4. The presence of an ethyl group on C-1 was evident by the triplet at δ 0.99 (*J* = 6.6 Hz) assigned to Me-3 and the multiplet at δ range 1.40–1.70 assigned to CH_2-2. At δ 1.02 appears a singlet assigned to Me-15. All ^{1}H and ^{13}C NMR spectroscopic data are listed in Tables 1 and 2, respectively, and are in agreement with structure **2**. The assignments of the signals were made with the aid of gHSQC and gHMBC experiments. NOESY experiments were performed to confirm the relative configuration of **2** and the observed correlations are shown in Fig. 1B. Compound **3** showed a similar ^{1}H NMR spectrum than **2**, but the main difference was observed at the signal assigned to H-4 that in **3** appears at δ 4.42 (brd, *J* = 3.5 Hz) instead of δ 4.70 as in **2**. This data, along with those from the ^{13}C NMR spectrum, indicated that **3** is the C-4 epimer of **2**. The difference observed for the W-type coupling constant value between H-4 and H-14 in both **2** and **3** is due to the conformation of the six-member ring in both compounds. The minimum energy conformation of **2**, which was obtained from the PCMODEL program, shows the six-member ring in a chair conformation with both H-4 and H-14α in the same plane, which justifies a large W-type coupling constant value.

Table 2
^{13}C NMR spectroscopic data for compounds **1**–**3** (50.3 MHz, $CDCl_3$, TMS as internal standard).

	1	2	3
C-1	37.15	38.64	40.65
C-2	26.70	25.24	25.80
C-3	59.24	19.41	20.11
C-4	99.83	99.55	99.55
C-5	37.73	39.54	41.21
C-6	32.57	38.05	42.87
C-7	38.40	42.75	45.73
C-8	77.36	85.57	84.16
C-9	34.43	42.64	42.75
C-10	31.15	27.64	27.96
C-11	37.85	138.86	138.86
C-12	177.49	169.12	169.12
C-13	20.05	117.52	118.31
C-14	10.27	59.88	65.49
C-15	19.99	21.48	19.41

Table 3
Dihedral angles and calculated and observed coupling constant values for **1.**

Position	θ (deg)	J_{calcd}(Hz)	J_{obs}(Hz)
1α, 2α	−61	3.1	4.0
1α, 2β	−179	12.3	11.0
1α, 10α	38	6.4	5.6
2α, 3α	54	2.7	4.2
2α, 3β	−64	1.3	1.7
2β, 3α	170	11.9	11.8
2β, 3β	52	5.1	4.8
6α, 7α	74	1.6	2.2
6β, 7α	−175	12.0	11.0
7α, 8α	−25	6.4	5.7
7α, 11α	30	8.3	9.0
8α, 9α	−57	4.6	6.6
8α, 9β	−171	10.8	8.6
9α, 10α	53	4.3	4.3
9β, 10α	166	11.8	10.7

However, the minimum energy conformation of **3** shows the six-member ring in a boat-like conformation with H-14β slightly out of the plane conformed by H-4β-C-4-O-C-14.

As mentioned above, seco-pseudoguaianolides are common compounds found in the genus *Hymenoxys*. Herz (1977a,b) has proposed that seco-pseudoguaianolides are categorized at the fourth level of biogenetic complexity (from 1 to 4). The biosynthesis of these compounds commences by a cyclization of either a germacrolide or melampolide precursor, which generates the guaianolide skeleton. After a number of structural rearrangements, the guaianolide is transformed into a pseudoguaianolide skeleton followed by a five-membered ring opening to produce the seco-pseudoguaianolide skeleton. Generally, the lower complexity level skeletons have broader distributions within the family than the higher complexity level skeletons. Particularly, compound **2** and **3** have the 3,4-seco-pseudoguaianolides skeleton, which is also termed 3,4-secohelenanolide because C-14 is α-oriented. It is proposed that a nucleophilic attack of the hydroxyl group at C-14 on the carbonyl group at C-4 generates the hemiacetal ring. Due to the co-occurrence of C-14 α- and β-oriented in the same plant, it is postulated that **1** and **2**–**3** arised by cyclization of separate *trans, trans*- and *cis,*

Table 4
^{1}H and ^{13}C NMR spectroscopic data for compounds **5**–**6** (200 and 50.3 MHz, $CDCl_3$, TMS int. std., δ values).

Atom	H		C	
	5	6	5	6
1	5.26 dd (4.2; 2.7)	5.32 dd (5; 1.6)	66.89	66.93
2	5.28 dd (4.2; 4.2)	5.35 dd (5)	66.61	66.66
3	5.14 dd (10.7; 4.2)	5.21 dd (10; 5)	70.48	70.47
4	5.25 dd (10.7; 9.2)	5.33 dd (10)	71.23	71.22
5	3.91 dd (10; 9.2)	3.96 dd (10)	69.88	69.91
6	5.13 dd (10; 2.7)	5.20 dd (10; 1.6)	68.33	68.36
OAc	1.92 s	1.99 s	169.20	169.25
	2.10 s	2.11 s	168.36	168.38
	2.04 s	2.17 s	167.57	167.59
			20.56	20.65
			20.56	20.59
			20.41	20.42
OiBu	2.57 hept (6.9)	2.53 hept (7)	174.76	172.81
	1.15 d (6.9)	1.16 d (7)	173.22	33.64
	1.16 d (6.9)	1.21 d (7)	33.59	18.64
	2.46 hept (7.3)		18.88	18.52
	1.07 d (7.3)		18.60	
	1.09 d (7.3)			
O-2-MeBu		2.47 sext (6.8)		174.85
		1.52 m		40.66
		1.73 m		26.37
		1.14 d (6.8)		16.62
		0.96 t (7.4)		11.46

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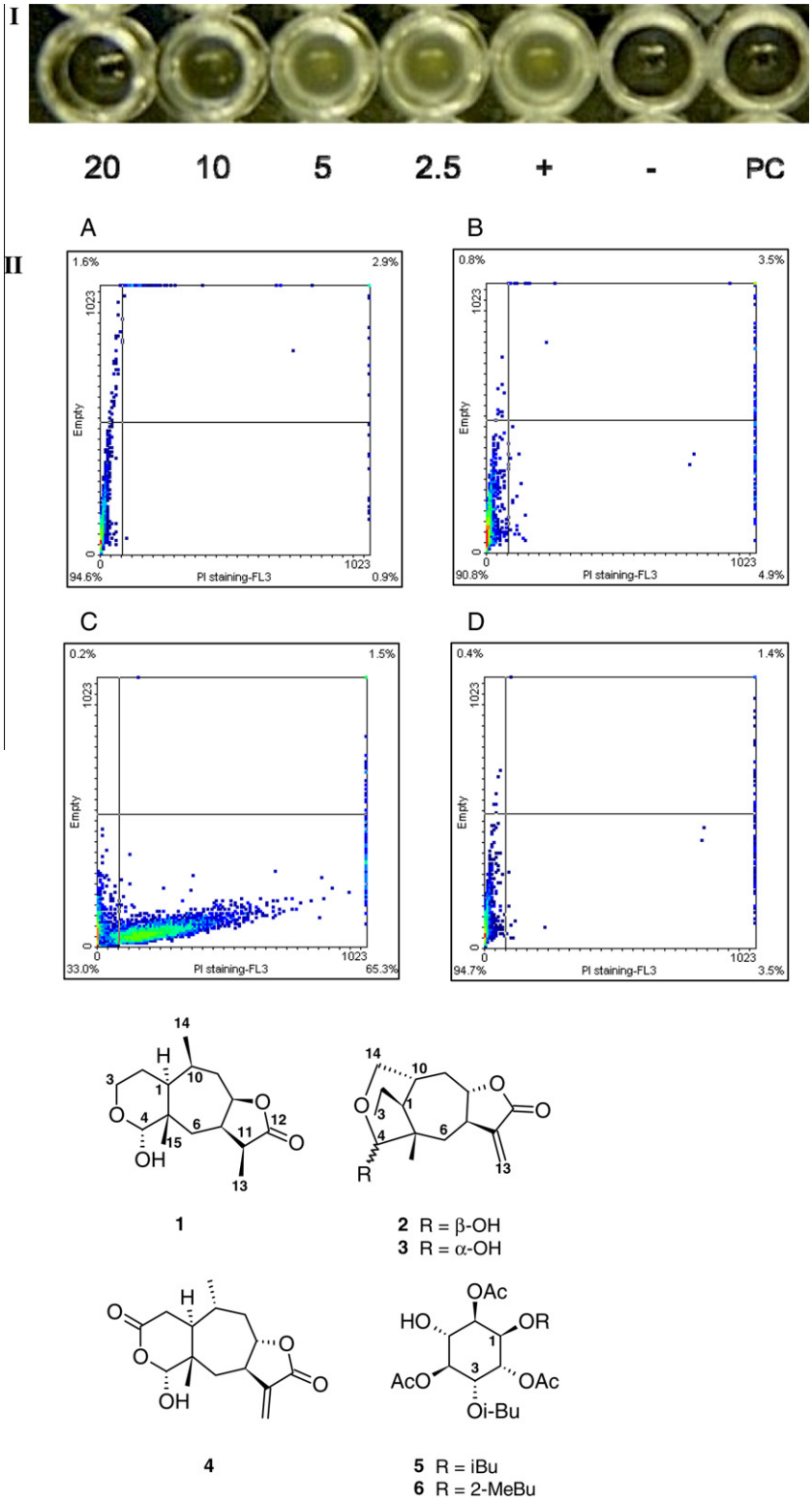

Fig. 2. MIC and cytotoxic effects of vermeerin (**4**). (I) Serial dilutions of vermeerin (**4**) were exposed to *Staphylococcus aureus*. Numbers indicate the concentration of the compound in μg/mL. "+", bacteria and 5% DMSO; "–" no bacteria added, "PC", positive control (gentamicin). (II) Determination of the cytotoxic effects on THP-1 cells of vermeerin using FACS analysis (A), DMSO (B), and H_2O_2 (C). No treated THP-1 cells (D) were used as negative control.

trans-1(10)-germacradiene precursors or their epoxide analogues respectively (Herz, 1977a).

Compound **5** was obtained as colorless oil. The IR spectrum showed absorptions for hydroxyl (3498 cm^{-1}) and ester groups (1731 cm^{-1} and 1739 cm^{-1}). The HRMS showed an $[M+H]^+$ ion peak at *m*/*z* 447.1874 (calcd for $C_{20}H_{31}O_{11}$ 447.1866) according to a molecular formula $C_{20}H_{30}O_{11}$. On the other hand, LRMS showed peaks at *m*/*z* 429 $[M+H-H_2O]^+$, 387 $[M+H-AcOH]^+$, and 359 $[M+H-C_4H_8O_2]^+$, indicating the presence of hydroxyl, acetate and a four-carbon atom ester, respectively. The ^{13}C NMR spectrum of **5** exhibited signals between δ 66.61 and 71.23 for six oxygenated carbon atoms, typical for a carbohydrate skeleton (Table 4).

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However, the absence of any anomeric carbon signals indicated that this substance was not a sugar. 2D COSY experiment established, on the basis of coupling patterns, the presence of an oxygenated cyclohexane ring suggesting that **5** was a *chiro*-inositol. The ^{1}H NMR spectrum showed three singlet signals at δ 1.92, 2.04, and 2.10 indicating the presence of three acetate residue, as well as, resonances at δ 2.57 (hept, J = 6.9 Hz) and 2.46 (hept, J = 7.3), which along with four methyl doublets between δ 1.07 and 1.16 indicated the presence of two isobutyryloxy residues. Additional signals were observed at δ 3.91 (dd, J = 10; 9.2 Hz) assigned to a α-oriented hydroxyl group, which is in an equatorial position from their coupling constant values. These also indicated that the ester side-chain on C-4 and C-6 are β-oriented. From the coupling constant values of all hydrogen attached to the oxygenated carbons of the cyclohexane ring, the relative stereochemistry was established. The position of all ester residues resulted from a thorough study of gCOSY, gHSQC, and gHMBC experiments, as well as, the assignment of both ^{13}C and ^{1}H NMR signals (Table 4).

Compound **6** differed from **5** in that a 2-methylbutyryloxy side chain replaced one of the isobutyryloxy groups as indicated by the presence of resonances at δ 2.47 (sext, J = 6.8 Hz), 1.73 (m), 1.52 (m), 1.14 (d, J = 6.8 Hz), and 0.96 (t, J = 7.4 Hz), and the presence of only one set of signals corresponding to an isobutyryloxy residue. In agreement with these facts, the molecular formula $C_{21}H_{32}O_{11}$, resulted from its HRMS, which showed a $[M+H]^+$ at m/z 461.2031 (calcd for $C_{21}H_{33}O_{11}$ 461.2023), while LRMS showed peaks at m/z 443 (100%) $[M+H-H_2O]^+$, 401 $[M+H-AcOH]^+$, 373 $[M+H-C_4H_8O_2]^+$, and 359 $[M+H-C_5H_{10}O_2]^+$, indicating the presence of hydroxyl, acetate, a four- and a five-carbon atom esters, respectively. All ^{1}H and ^{13}C NMR signals assignments were made with the aid of gCOSY, gHSQC, and gHMBC experiments.

All of the six compounds isolated from this plant were tested against a panel of bacterial and fungal pathogen strains as detailed in the Section 4. Only vermeerin (**4**) showed activity specific against *Staphylococcus aureus* with a MIC of 10 μg/mL (Fig. 2I), while the rest of the compounds showed no activity against all of the tested pathogens (data not shown).

Since *S. aureus* constitutes the leading cause of skin, lower respiratory tract and blood stream infections worldwide (Diekema et al., 2001), it was tested as to whether vermeerin (**4**) is toxic to macrophages. Results showed that a similar percentage (>90%) of viable cells were measured when the bioactive concentration of the compound (10 μg/mL) were analyzed by flow cytometry (Fig. 2II-A). Similar results were observed when DMSO (the solvent) or non-treated THP-1 cells (negative control) were subjected to FACS analysis. In contrast, only 33% of macrophages survived (Fig. 2II-C) when were exposed to H_2O_2 (positive control), suggesting that vermeerin was not toxic to the human-derived macrophages THP-1. Since vermeerin was isolated from a weed, these results encourage the development of a new treatment against *S. aureus* infections.

3. Conclusions

In this study the isolation and identification of six compounds from *H. robusta* are reported. These metabolites include three new seco-pseudoguaianolides(**1**–**3**), two new inositols(**5**–**6**), and the known dilactone sesquiterpene vermeerin (**4**). The identities of these new molecules are in agreement with identities of molecules isolated from other species of the same genus. The variability and complexity of these metabolites in *Hymenoxys* suggest that this genus is more evolved than other genera of the Asteraceae family. In this study, it was found that from the six isolated compounds tested, only vermeerin showed antimicrobial activity against *S. aureus*. No cytotoxic activities against the human-derived macrophages THP-1 were observed reflecting promising applications of this compound to be considered as a potential treatment against *S. aureus*-associated infections.

4. Experimental

4.1. General experimental procedures

Column chromatography (CC) was carried out on Merck Si gel 60 (70–230 mesh ASTM) or (230–400 mesh ASTM). For separation of mixtures, HPLC with a differential refractometer detector was used. Columns employed were: (A) Beckman C-8 (5 μm, 10 × 250 mm) and (B) Beckman C-18 (5 μm, 10 × 250 mm). Retention times (t_R) were measured from the solvent peak.

UV spectra were measured on a Varian Cary 100 spectrometer, whereas IR spectra were obtained on a BioRad FTS135 spectrometer, with mass spectra acquired with a JEOL MStation JMS 700 high-resolution mass spectrometer in the fast atom bombardment (FAB) mode by using *m*-nitrobenzyl alcohol (NBA) as the matrix. NMR spectroscopic measurements were recorded on a Varian Associates Gemini spectrometer operating at 200 MHz for hydrogen and 50.3 MHz for carbon, using 5 mm sample tubes in deuterated chloroform solution containing tetramethyl silane (TMS) as internal standard.

4.2. Plant material

Specimens of *H. robusta* were collected at Jujuy province, Humahuaca Department, Locality of Uquía (2800 m above sea level), Argentina, during January 2006. A voucher specimen was deposited at the herbarium of the Instituto Miguel Lillo, Tucumán, Argentina under the register number Fortuna s/n (LIL416346), after identification of the plant material by Dr. Alberto Slanis.

4.3. Extraction and isolation

Air-dried aerial parts (937 g) of *H. robusta* were extracted with $CHCl_3$ (5 L) at room temperature for 2.5 days. The extract was evaporated at reduced pressure (40 °C) to give a crude extract 52.8 g, which was suspended in EtOH (430 mL) at 60 °C, diluted with H_2O (325 mL) and extracted successively with petroleum ether 60–80° (3 × 350 mL) and $CHCl_3$ (3 × 350 mL). Evaporation of the $CHCl_3$ extracts at reduced pressure gave residue 16.5 g and a portion (14.7 g) was subjected to silica gel CC, 210 fractions were collected after elution with $CHCl_3$ and subsequently with increasing volumes of EtOAc (0–100%), and MeOH (0–100%). The resulting fractions were monitored by TLC and grouped according to their profile.

Fractions 102–108 (201 mg) were combined and processed by HPLC (column A using a mixture of MeOH–H_2O 1:1), and vermeerin (**4**) (27 mg, t_R 77 min) were obtained. Fractions 115–119 (306 mg) were combined and processed by HPLC (column B using a mixture of MeOH–H_2O 3:2), and 4 fractions were collected. Fraction 4 afforded **6** (37.6 mg, t_R 43 min), while the rest of the fractions was rechromatographed individually using the same column, but using a different mixture of solvents (MeOH–H_2O 4:3). Fraction 2 (t_R 15 min) gave of **1** (3 mg, t_R 11 min) and a mixture of the sesquiterpene lactones **2** and **3** (10.2 mg, t_R 30 min). 36.4 mg (t_R 35 min) of **5** was also recovered from Fraction 3.

4.3.1. Robustolide (*1*)

Yellow oil; $[\alpha]_{589}^{20} = -37.4$ (c = 0.2, $CHCl_3$); IR ($CHCl_3$) 3409, 1769 cm^{-1}; for ^{1}H ($CDCl_3$, 200 MHz) and ^{13}C ($CDCl_3$, 50 MHz) NMR spectroscopic data, See Tables 1 and 2. LRMS m/z 269 $[M+H]^+$ (44), 251 (100), 154 (94), 136 (85), 107 (40), 91 (46), 77

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(47), 57 (41); HRMS *m/z* 269.1748 $[M+H]^+$ (calcd for $C_{15}H_{25}O_4$ 269.1753).

4.3.2. Hymenolides (**2** and **3**)

Yellow oil; IR ($CHCl_3$) 1746, 1650 cm^{-1}; for ^1H ($CDCl_3$, 200 MHz) and ^{13}C (CDC_{13}, 50 MHz) NMR spectroscopic data, See Tables 1 and 2. LRMS *m/z* 267 $[M+H]^+$ (4), 249 (5), 154 (100), 136 (73), 107 (21), 89 (20), 77 (16), 57 (10); HRMS *m/z* 267.1606 $[M+H]^+$ (calcd for $C_{15}H_{23}O_4$ 267.1596).

4.3.3. 2,4,6-tri-O-Acetyl-1,3-di-O-isobutyryl-chiro-inositol (**5**)

Colorless oil; $[\alpha]_{589}^{20} = -37.4$ (c = 0.6, $CHCl_3$); IR ($CHCl_3$) 3498; 1731 and 1739 cm^{-1}; for ^1H ($CDCl_3$, 200 MHz) and ^{13}C ($CDCl_3$, 50 MHz) NMR spectroscopic data, see Table 4. LRMS *m/z* 447 $[M+H]^+$ (11), 429 (71), 387 (43), 359 (28), 317 (9), 137 (15), 109 (14), 71 (100), 55 (15); HRMS *m/z* 447.1874 $[M+H]^+$ (calcd for $C_{20}H_{31}O_{11}$ 447.1866).

4.3.4. 2,4,6-tri-O-Acetyl-3-O-isobutyryl-1-O-2-methylbutyryl-chiro-inositol (**6**)

Colorless oil; IR ($CHCl_3$) 3500; 1738 and 1730 cm^{-1}; for ^1H ($CDCl_3$, 200 MHz) and ^{13}C ($CDCl_3$, 50 MHz) NMR spectroscopic data, see Table 4. LRMS *m/z* 461 $[M+H]^+$ (11), 443 (100), 401 (65), 359 (30), 317 (10), 169 (10), 109 (15), 85 (60), 71 (36), 57 (34); HRMS *m/z* 461.2031 $[M+H]^+$ (calcd for $C_{21}H_{33}O_{11}$ 461.2023).

4.4. Microbial strains and culture media

The following Gram negative microorganisms were evaluated: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumonia* (ATCC 10031). Amongst Gram positive microorganisms *Mycobacterium smegmatis* (strain mc^2155, ATCC 700084), and *S. aureus* (ATCC 25923). *Candida albicans* (ATCC 14053), *Cryptococcus neoformans* (kindly donated by Dr. James Kronstad, Michael Smith Laboratories, UBC, Canada), *Aspergillus niger* (ATCC 32656), and *Trychophyton mentagrophytes* were used as representatives of fungal pathogens. All organisms were grown in brain heart infusion (BHI) broth (BD, CA) with the exception of *M. smegmatis* and fungi, which were grown in Middlebrook 7H9 supplemented with 10% OADC and Sabouraud Dextrose broths respectively. Solid media was obtained by adding 1.5% (w/v) agar.

4.5. Antimicrobial test

Compounds were dissolved in DMSO at a final concentration of 10 mg/mL, and serial dilutions were prepared using the same solvent. The published microdilution method for estimation of minimum inhibitory concentration (MIC) values was carried out to evaluate the antimicrobial activity. The MIC was determined on 96-well microdilution plates and according to published protocols (Standards, 1997, 2003). MICs were determined by incubating the organisms in 96-well microplates for 24 h at 37 °C with the exception of fungi, which were cultured at 28 °C. *M. smegmatis* was cultured at 37 °C for 48 h. Microorganisms were exposed to serial dilutions of the compounds keeping a final concentration <5% in each well to avoid DMSO toxicity. Endpoints were determined when no turbidity in the well was observed. The antibacterial activities of the compounds were compared to methicillin (50 μg/mL), and gentamicin (50 μg/mL), whilst amphotericin B (20 μg/mL) was used as positive control for fungi. DMSO (5%) included in the broth and untreated inoculum were used as controls. All assays were carried out in triplicate.

4.6. Measurement of cytotoxicity

Monocytic cell line THP-1 (ATCC 202) was cultured in RPMI 1640 (Hyclone, UT) supplemented with 5% fetal calf serum (Hyclone, UT), and 2 mM L-glutamine (StemCell Technologies, BC, Canada). Cells were dispensed in 96-well microplates at a density of 3×10^5/well. Concentrations showing antimicrobial activities were tested for each compound, in a final volume of 200 μl/well. Microplates were incubated at 37 °C in a humidified atmosphere supplemented with 5% CO_2 for 24 h. THP-1 cells treated for 6 h with 5% H_2O_2 were used as positive controls, while untreated cells and cells treated with DMSO were used as negative control. The toxicity of the compounds was measured by staining the monocytes with propidium iodide (PI) (Sigma) and according to published protocols (Pick et al., 2004). PI emission was detected at 610–625 nm using the FL3 gate of a BD FACS Vantage SE Turbo sort cell sorter (BD, ON, Canada).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.09.001.

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