



Ethnopharmacological communication

Sesquiterpene lactones from *Gynoxys verrucosa* and their anti-MRSA activity

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ABSTRACT

Ethnopharmacological relevance: Because of its virulence and antibiotic resistance, *Staphylococcus aureus* is a more formidable pathogen now than at any time since the pre-antibiotic era. In an effort to identify and develop novel antimicrobial agents with activity against this pathogen, we have examined *Gynoxys verrucosa* Wedd (Asteraceae), an herb used in traditional medicine in southern Ecuador for the treatment and healing of wounds.

Materials and methods: The sesquiterpene lactones leucodine (**1**) and dehydroleucodine (**2**) were extracted and purified from the aerial parts of *Gynoxys verrucosa*, and their structure was elucidated by spectroscopic methods and single-crystal X-ray analysis. The *in vitro* anti-microbial activity of *Gynoxys verrucosa* extracts and its purified constituents was determined against six clinical isolates including *Staphylococcus aureus* and *Staphylococcus epidermidis* strains with different drug-resistance profiles, using the microtiter broth method.

Results: Compound **1** has very low activity, while compound **2** has moderate activity with MIC₅₀s between 49 and 195 µg/mL. The extract of *Gynoxys verrucosa* has weak activity with MIC₅₀s between 908 and 3290 µg/mL.

Conclusions: We are reporting the full assignment of the ¹H NMR and ¹³C NMR of both compounds, and the crystal structure of compound **2**, for the first time. Moreover, the fact that compound **2** has antimicrobial activity and compound **1** does not, demonstrates that the exocyclic conjugated methylene in the lactone ring is essential for the antimicrobial activity of these sesquiterpene lactones. However, the weak activity observed for the plant extracts, does not explain the use of *Gynoxys verrucosa* in traditional medicine for the treatment of wounds and skin infections.

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1. Introduction

Staphylococcus aureus is a devastating bacterial pathogen capable of causing a broad array of infections in both humans and animals. The ability to treat these infections is compromised by the continued emergence of antibiotic-resistant strains. This includes strains resistant to methicillin which in recent years have become predominant even among community-acquired isolates (Deurenberg and Stobberingh, 2009). For example, in 2005, invasive infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) affected 94,630 patients in the US alone, resulting

in approximately 18,650 deaths – exceeding the national mortality rates for HIV/AIDS (Klevens et al., 2007).

Most ominous is the appearance of high-level vanA-mediated vancomycin resistance even among methicillin-resistant *Staphylococcus aureus* (MRSA). Although these isolates remain rare (Zhu et al., 2008), this is likely to change given, the increasing reliance on vancomycin as a consequence of the continued emergence of MRSA. New agents with activity against these strains exist, but resistance to these has also begun to appear (Lee, 2008). This emphasizes the urgent need to identify and develop novel antimicrobial agents with activity against MRSA. One possible source of such agents is natural products derived from various biological sources including plants. In this context it has been recently emphasized that natural products, by acting through different mechanisms from that of conventional antibiotics, could be of clinical value in the treatment of resistant bacteria (Simoes et al., 2009).

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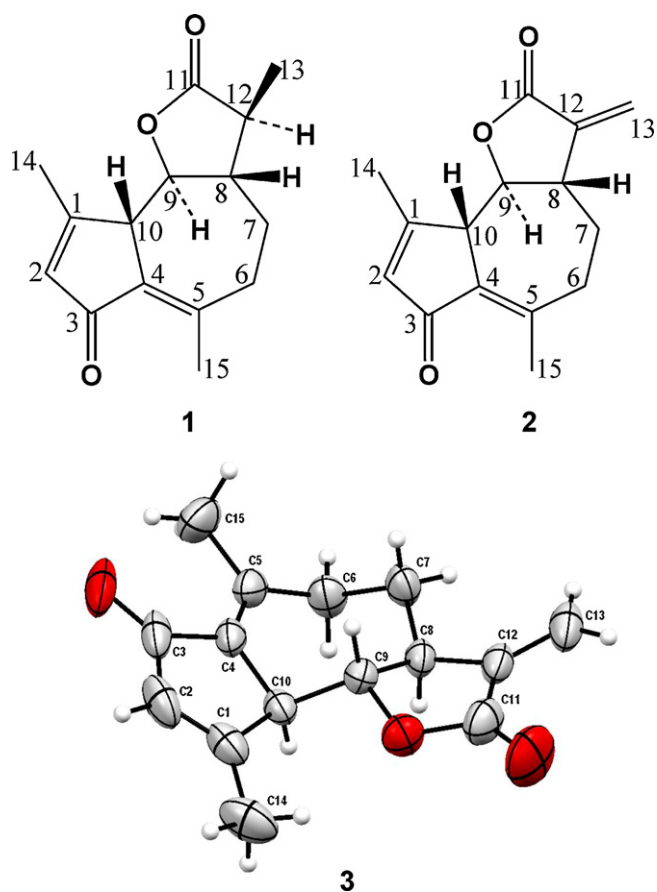


Fig. 1. Structures of leucodine (**1**), dehydroleucodine (**2**) and 3-D-ORTEP projection of the X-ray crystal structure of dehydroleucodine with 50% probability ellipsoids (**3**).

Gynoxys verrucosa is a shrub belonging to the Senecionea tribe of the Asteraceae family. The aerial parts of this plant, commonly known as *guangalo*, are used in traditional medicine in southern Ecuador for the treatment of skin infections and wound healing by direct application to the skin (Tene et al., 2007).

Phytochemical investigation of the ethyl-acetate extract from the aerial parts of *Gynoxys verrucosa* led to the isolation of two known sesquiterpene lactones, of the guainolide group, leucodine (**1**) and dehydroleucodine (**2**) (Fig. 1). These compounds have not been previously reported in this plant species but they have been reported in other members of the Asteraceae family, including *Artemisia douglasiana* Besser (Wendel et al., 2008), *Picris koreana* Vorosch. (Michalska et al., 2007), *Warionia saharae* Benthem ex Coss. (Hilmi et al., 2003), *Kaunia rufescens* (P.W. Lund ex DC.) R.M. King & H. Rob. (Rucker et al., 1997), *Achillea collina* (Becker ex Rchb. f.) Heimerl (Trendafilova et al., 2006) and *Stevia pilosa* Lag. (Martínez and Muñoz-Zamora, 1988). However, the full assignment of the ^1H NMR and ^{13}C NMR of either the compound or the crystal structure of compound **2** have not been reported. Although, the biological activity of compound **2** has been demonstrated in various biological systems (Wendel et al., 2008; Vega et al., 2009), its activity against Gram-positive bacteria, in general, or *Staphylococcus* sp., in particular, has not been previously reported.

2. Materials and methods

2.1. General experimental procedures

Melting points were measured with a capillary melting point apparatus and are uncorrected. IR spectra and optical rotation

were measured on a Magna IR 550 spectrometer and an Autopol III (Rudolph) polarimeter, respectively. UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. NMR spectra were recorded in CDCl_3 , at 25°C , on a Varian Unity NMR spectrometer, operating at 500 MHz for ^1H and 125 MHz for ^{13}C spectra, equipped with a Nalorac 3 mm inverse detection probe with a z axis gradient coil. Each sample consisted of ca. 5 mg of compound dissolved in 1 mL of CDCl_3 containing TMS as an internal reference. All 1D and 2D spectra were acquired and processed with standard Varian software. Mass spectral data were obtained in an Agilent 5975 GC/MSD instrument operating under EI conditions at 70 eV. Column chromatography was carried out using 60–230 mesh silica gel. Preparative TLC was performed on pre-coated silica gel 60 F₂₅₄ plates (0.2 mm thick, Merck).

2.2. Plant material

The aerial parts of *Gynoxys verrucosa* were collected in June 2007 in the locality of Yangana, in the Loja province of Ecuador. Voucher specimens (PPN-as-11) are deposited at the Herbarium of the Applied Chemistry Institute of the Universidad Técnica Particular de Loja, Loja Ecuador, and at the Herbarium Reynaldo Espinoza of the Universidad Nacional de Loja.

2.3. Extraction and isolation

The air-dried plant material (200 g) was extracted with ethyl acetate (dynamic maceration for 5 h) at room temperature and concentrated under reduced pressure. The extract (14 g) was filtered through a reverse phase C₁₈ column (LiChroprep Merck 25–40 μm) with a mixture MeOH/H₂O 85:15, for the removal of chlorophylls. The filtrate (7 g) was fractionated by column chromatography using a hexane–EtOAc gradient. Compound **2** was eluted in the hexane:EtOAc (85:15) fraction (0.7 g) and recrystallized from EtOAc as a white crystalline solid. Compound **1** was isolated from the mother liquid using preparative TLC hexane: EtOAc (70:30) and recrystallized from EtOAc.

2.4. Physical and spectroscopic data

Leucodine (**1**): colorless crystals (EtOAc), mp $199\text{--}200^\circ\text{C}$; $[\alpha]_{\text{D}}^{25} +61.7$ (c 0.41, CH_2Cl_2); UV (MeOH) λ_{sh} (log ϵ) 214 (3.78) nm, λ_{max} (log ϵ) 256 (4.22) nm; IR (KBr) ν_{max} 2940, 1780, 1686, 1637, 1620 cm^{-1} ; EI-MS: m/z (rel. int.) 246 (100), 231 (10), 217 (26), 173 (31), 159 (16), 145 (19), 135 (14), 115 (9), 105 (16), 91 (42), 77 (19); ^1H NMR (CDCl_3 , 500 MHz) see Table 1; ^{13}C NMR (CDCl_3 , 125 MHz) see Table 1.

Dehydroleucodine (**2**): colorless crystals (EtOAc), mp $128\text{--}130^\circ\text{C}$; $[\alpha]_{\text{D}}^{25} +48.98$ (c 1.42, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 216 (3.91) nm, 256 (4.20) nm; IR (KBr) ν_{max} 3098, 2970, 1780, 1681, 1631, 1615 cm^{-1} ; EI-MS: m/z (rel. int.) 244 (100), 183 (13), 173 (13), 159 (15), 145 (18), 133 (10), 115 (13), 91 (46), 77 (22), 53 (22); ^1H NMR (CDCl_3 , 500 MHz) see Table 1; ^{13}C NMR (CDCl_3 , 125 MHz) see Table 1.

X-ray data of compound **2**: crystal data: C₁₅H₁₆O₃. Mw = 244.29, orthorhombic, space group P2₁2₁2₁, $a = 7.5784(5)\text{Å}$, $b = 11.1203(7)\text{Å}$, $c = 15.3702(11)\text{Å}$, $V = 1295.31(15)\text{Å}^3$, $Z = 4$, crystal dimensions: 0.22 mm \times 0.37 mm \times 0.6 mm, $T = 223\text{K}$ Rigaku SCXmini system, Mercury 2 CCD detector, Mo-K α radiation ($\lambda = 0.71075\text{Å}$), 9029 total measured reflections, 2969 unique measured reflections, $R_{\text{sym}} = 0.025$, exposure time: 10 s/image, total collection time: 4 h 12 min, crystal-to-detector distance: 50.1 mm, 2θ swing: 30.0° , number of scans: 2, data images: 360, $2\theta_{\text{max}} = 55.0^\circ$. R_{factor} (all unique reflections) = 0.054. CCDC 730479 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Cry-

Table 1
NMR spectroscopy data at 500 MHz for ¹H and 125 MHz for ¹³C for compounds **1** and **2**.

Position	Leucodine (1)		Dehydroleucodine (2)	
	δ _H	δ _C	δ _H	δ _C
1	6.16 (q, 1.2)	169.92	6.17 (q, 1.2)	169.4
2		135.55		135.8
3		195.91		195.7
4		131.85		131.9
5		152.10		151.8
6	2.42 (ddd, 14.5, 12.5, 2)	37.55	2.52 (ddd, 1.5, 12.5, 2)	37.3
7	2.33 (ddd, 14.5, 6, 2)	25.98	2.39 (ddd, 14.5, 6, 2)	24.5
8	1.99 (m)	56.37	2.22 (ddd, 14, 6, 3, 2)	53.1
9	1.36 (dddd, 14, 12, 10, 2)	84.20	1.44 (dddd, 14, 12, 10, 2)	84.4
10	1.96 (m)	52.56	2.89 (ddd, 12, 10, 3)	53.0
11	3.62 (t, 10.0)	177.54	3.62 (t, 10)	53.1, 169.4
12	3.41 (bd, 10)	41.13	3.52 (bd, 10)	169.4
13	2.25 (dq, 12.5, 6.9)	12.28	6.18 (d, 3.3)	169.1, 138.5, 53.1
14	1.27 (d, 6.9, 3H)	19.80	5.47 (d, 3.3)	169.1, [138.5], 53.1
15	2.29 (t, 1.2, 3H)	21.61	2.33 (t, 1.2, 3H)	135.8, 53.0, 169.4
	2.44 (bs, 3H)		2.44 (bs, 3H)	151.9, 131.9, 37.3

^a Figures in square brackets denote weak long range correlations.

tallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033; e mail: deposit@ccdc.cam.ac.uk).

2.5. Antimicrobial activity

2.5.1. Bacteria and culture conditions

Six staphylococcal isolates were used in the determination of bacteriostatic activity. Two strains of *Staphylococcus epidermidis* (UAMS-302 and UAMS-1037) and four strains of *Staphylococcus aureus* (UAMS-1, UAMS-1625, UAMS-1864, and UAMS-1865) were included in the study. The antibiotic resistance profile for each strain is detailed in Table 2. Staphylococcal isolates were grown in tryptic soy broth (TSB) overnight at 37 °C and diluted to the equivalent of OD_{600nm} = 0.05 in TSB for MIC₅₀ testing.

2.5.2. Determination of antibiotic resistance profile

Staphylococcal isolates included in this study were examined for their antibiotic resistance profile using the disc diffusion method following established protocols (Isenberg, 2004). Briefly, Mueller-Hinton agar was seeded with bacteria and antibiotic discs: 1 µg oxacillin, 10 µg ampicillin, 5 µg ciprofloxacin, 2 µg clindamycin, 30 µg cefazolin, 15 µg erythromycin, 10 µg gentamicin, 30 µg kanomycin, 30 µg neomycin, 10 units penicillin, 25 µg sulfamethoxazole/trimethoprim, 30 µg tetracycline and 30 µg vancomycin (Becton Dickinson, BBL Antibiotic Susceptibility Discs, NJ, USA) were applied to the agar. Following incubation, the diameter of the zone of inhibition was measured and compared to the antibiotic kit's susceptibility chart for *Staphylococcus aureus* and *Staphylococcus epidermidis*.

2.5.3. Determination of MICs

Minimum inhibitory concentrations (MICs) were determined by the microtiter broth method (Amsterdam, 1996) in sterile flat-bottom 96-well polystyrene plates. Bacteria were suspended in TSB and added to microtiter plates. Serial dilution techniques were employed to examine the activity of compounds **1** and **2** (purity >99%), and extract at concentrations ranging from 3 to 1000 µM. In addition, negative (growth) controls (cells + TSB), vehicle controls (cells + TSB + DMSO), and positive controls (TSB + antibiotic; vancomycin or oxacillin) were included. All tests were performed in triplicate. Microtiter plates were placed in a 37 °C incubator on a shaker for 18 h. Optical density readings (λ = 600 nm) were taken using a Biotek microplate reader at 0 and 18 h post-inoculation. Results are reported as the MIC₅₀ and MIC₉₀ for growth at 18 h post-inoculation.

3. Results and discussion

From the ethyl acetate extract of *Gynoxys verrucosa*, two sesquiterpene lactones were isolated and identified by spectroscopy methods and by single-crystal X-ray analysis. Table 1 shows the NMR analysis of both isolated compounds.

The X-ray crystal structure of compound **2**, was established and reported for the first time for this molecule, and is fully consistent with the previously reported structure (Wendel et al., 2008). The molecular structure of **2** is illustrated in Fig. 1, the crystal structure shows that both H-8 and H-10 are below the approximate plane of the seven membered ring, while H-9 is above the plane, hence, the configuration is confirmed as being 8α-H, 9β-H, and 10α-H. The seven-membered ring shows an approximated chair conformation with the atoms C-4, C-5, C-6, and C-10 approximately coplanar, and the atoms C-7, C-8, and C-9 above that plane. The full assignment of the ¹H NMR and ¹³C NMR spectra for compounds **1** and **2** are

Table 2
MIC₅₀ and MIC₉₀ values for compounds **1**, **2**, and the ethyl acetate extract of *Gynoxys verrucosa* against various strains of methicillin resistant *Staphylococcus aureus* (MRSA), methicillin sensitive *Staphylococcus aureus* (MSSA), methicillin resistant *Staphylococcus epidermidis* (MRSE), and methicillin resistant *Staphylococcus epidermidis* (MSSE).

Organism	<i>Staphylococcus aureus</i> (UAMS-1625)	<i>Staphylococcus aureus</i> (UAMS-1864, ATCC ^a 33593)	<i>Staphylococcus aureus</i> (UAMS-1865, NRS ^b 385)	<i>Staphylococcus aureus</i> (UAMS-1, ATCC ^c 49230)	<i>Staphylococcus epidermidis</i> (UAMS-1037, NRS ^b 101, ATCC ^c 35984)	<i>Staphylococcus epidermidis</i> (UAMS-302, O-47 ^c)
Phenotype Source	MRSA Brain abscess (Sifri et al., 2007)	MRSA Bloodstream	MRSA Bloodstream	MSSA Osteomyelitis (bone)	MRSE Catheter sepsis	MSSE Unknown
Antibiotic resistance profile ^d	AMP; CIP; ERY; KAN; LVX; NEO; OX; PEN	AMP; CLI; ERY; GM; KAN; OX; PEN; SXT; TET	AMP; CIP; CLI; CZ ^e ; ERY; GM; KAN; LVX; OX; PEN; SXT; TET	AMP; PEN	AMP; CIP; CLI; ERY; GM; KAN; LVX ^e ; OX; PEN	AMP; PEN
MIC ₅₀ (μg/mL)	908	1460	3290	980	2190	1300
Extract	>246	>246	>246	>246	>246	>246
1	49	49	147	98	195	195
2	36	26	92	3	19	0.08
OX	2.7	3.2	4	3.2	2.8	2.3
VAN						
MIC ₉₀ (μg/mL)	450	280	400	440	680	490
2	48	80	132	5	88	0.75
OX	4.9	4.7	5	4.8	4.6	4.5
VAN						

^a ATCC: American Type Culture Collection.

^b NRS: network for antibiotic resistant *Staphylococcus aureus*.

^c O-47 was kindly provided by the F. Götz's collection of clinical isolates from Germany.

^d AMP: ampicillin; CIP: ciprofloxacin; CLI: clindamycin; CZ: ceftazidim; ERY: erythromycin; GM: gentamicin; KAN: kanamycin; LVX: levofloxacin; NEO: neomycin; OX: oxacillin (methicillin); PEN: penicillin; SXT: sulfamethoxazole/trimethoprim; TET: tetracycline; VAN: vancomycin.

^e Intermediate resistance.

reported for the first time and are fully consistent with the proposed structures and the X-ray crystal structure of compound **2**. For compound **1** we observed a large (anti) H-8/H-12 coupling, a ROESY peak between H-12 and H-9 indicating that these hydrogens are on the same face and a ROESY peak between C(13)H₃ and H-8 indicating that these hydrogens are also on the same face.

The isolated constituents were tested *in vitro* against six staphylococcal isolates strains, compound **2** was found to have antimicrobial activity against all staphylococcal isolates, including four methicillin-resistant strains. The antimicrobial activities and the characteristics of the strains tested are presented in Table 2, and range from 49 to 195 μg/mL for the MIC₅₀ and 280–680 for the MIC₉₀. Although, the MRSA activity of compounds **2** is not very high, these results suggest its potential use as a template for further development. Furthermore, the significant activity of compound **2** against the Gram-negative *Helicobacter pylori* recently reported by Vega et al. (2009) suggests that this compound has a wide spectrum of antimicrobial activity. The fact that compound **2** showed activity against clinical isolates from brain abscesses, suggest that this compound may have a potential clinical use in this area. This is relevant because Staphylococcal brain abscesses are difficult to treat.

Although, no toxic effects have been reported for dehydroleucodine or *Gynoxys verrucosa*, caution should be advised, given that some sesquiterpene lactones have been reported to produce toxic effects to humans and animals, including contact dermatitis and neurotoxicity (Schmidt, 1999).

Compound **2** is a non-ionizable molecule, has a molecular weight smaller than 300, and showed the highest activity against two MRSA clinical isolates resistant to a broad range of antibiotics. All of these facts suggest that compound **2** may have potential for clinical applications as an antimicrobial or may serve as a template for the development of more potent antimicrobials. The establishment of the X-ray structure of compound **2** (Fig. 1) should assist in such efforts.

At a maximum test concentration of 1 mM, compound **1** showed very low level of activity, ranging from 0 to 5% inhibition against strains of *Staphylococcus aureus* and 5% and 33% inhibition for the strains of *Staphylococcus epidermidis*. Thus, these results demonstrate that the exocyclic conjugated methylene in the lactone ring is essential for the antimicrobial activity of these sesquiterpene lactones. These results are in contrast with the previously reported assertion that the exocyclic α-β-unsaturated methylene moiety is not required for the antimicrobial activity of the sesquiterpene lactones (Lee et al., 1977).

It is significant that three related sesquiterpene lactones structurally similar to compound **2** (arnicolide C, xanthatin and a sesquiterpene ketolactone) have also been found to have significant antimicrobial activity against MRSA and MSSA (Gibbons, 2004). Compound **2** and these compounds have, in addition to the carbonyl of the lactone ring, a carbonyl in the opposite side of the cycloheptane ring, suggesting that perhaps a secondary hydrogen binding point is necessary for the activity.

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