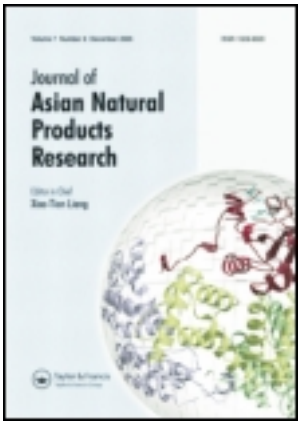

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Diterpenoids from the freshwater green algae *Rhizoclonium hieroglyphicum* with antibacterial activity

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Three new isopimarane diterpenes 7β-hydroxy-19α-methylmalonyloxy-isopimara-8(14),15-diene (**1**), 7β-hydroxy-14-oxo-isopimara-8(9),15-dien-19oic acid (**2**), and 7β-hydroxy-14-oxo-19α-methylmalonyloxy-isopimara-9(11),15-diene (**3**) in addition to the known compounds isopimaric acid (**4**), 7oxo-13-*epi*-pimara-14,15-dien-18oic acid (**5**), 7oxo-13-*epi*-pimara-8,15-dien-18oic acid (**6**), and 6β-hydroxyisopimaric acid (**7**) were isolated from the hexane extract of *Rhizoclonium hieroglyphicum*. The structures of compounds **1**–**7** were established by 1D and 2D NMR techniques. The isolated diterpenoids were screened for antimicrobial activity against gram-positive and gram-negative bacteria and yeast strains.

Keywords: *Rhizoclonium hieroglyphicum*; antimicrobial; isopimarane diterpenoids

1. Introduction

Rhizoclonium hieroglyphicum, commonly known as 'xochitl,' is traditionally used to cure many diseases, such as fever and skin diseases, in the forms of decoctions and infusions [1]. Previously, various components including saturated and unsaturated fatty acids having antimicrobial effect were reported from *R. hieroglyphicum* [2]. (*Z*)-9-octadecenamide was found to be the major component [3].

The aim of this study was to establish a deeper insight into the phytochemical constituents of *R. hieroglyphicum* and into their potential activity against gram-positive and gram-negative bacteria and fungal strains. Hexane extract of *R. hieroglyphicum* was chosen for detailed investigation, which led to the isolation and structural elucidation of three new diterpenoids (**1**–**3**) and four known compounds (**4**–**7**). Furthermore, the structure of diterpenoids was established using NMR. We herein report the antimicrobial activity of isolated diterpenoids and their structural determination.

2. Results and discussion

Hexane extract of *R. hieroglyphicum* was fractionated by chromatography on silica gel and Sephadex LH-20 to afford seven diterpenoids. The structures of compounds **1**–**7** were established by 1D and 2D NMR experiments and by the comparison of their physical and spectroscopic data with those previously reported in the literature. The main constituent of the extract was the known 6β-hydroxyisopimaric acid [4]. In addition, other known compounds

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isopimaric acid (**4**) [5], 7oxo-13-*epi*-pimara-14,15-dien-18oic acid (**5**) [6], and 7oxo-13-*epi*-pimara-8,15-dien-18oic acid (**6**) [6] were also isolated. Their structures were identified by comparing their IR and NMR spectral data with those published.

The structure of the new diterpenoids was established as follows. Compound **1** gave an accurate ion peak at *m/z* 404.2563 corresponding to the molecular formula $C_{24}H_{36}O_5$ (calcd 404.2576), which was also in agreement with ^{13}C NMR and DEPT data. The ^{13}C NMR (Table 1) spectrum indicated the presence of 24 unique carbon atoms assigned to four methyl, nine methylene, five methine and six quaternary (including two carbonyl groups and two double bonds) carbons. The presence of these groups was confirmed by its IR absorption bands (3400, 1691, and 1750 cm^{-1}). The typical resonances at δc 70.4 (C—OH), 138.9, 136.5 (C═C), 142, 115.3 (C═C), and 169.7 (C═O) are also displayed in the ^{13}C NMR spectrum. Interpretation of the ^{1}H and ^{13}C NMR spectral data suggested that **1** possessed an isopimarane skeleton [7].

The ^{1}H NMR spectrum showed three tertiary methyl signals at δ 1.12, 1.24, and 0.89, a typical ABX system of a vinyl side chain from signals at 5.82 (1H, dd, $J = 10.5$, 17.4 Hz, H-15), 4.95 (1H, dd, $J = 1.9$, 10.5 Hz, H-16), 5.10 (1H, dd, $J = 1.9$, 17.5 Hz, H-16), and a narrow triplet at δ 4.62 (1H, t, $J = 1.5$ Hz, H-7α) as indicative of the presence of a secondary β-hydroxyl group at C-7. Similar compounds with a C-7β hydroxyl group and a double bond at $\Delta^{8(14)}$ were previously isolated from *Aralia cordata* and *A. racemosa* and showed comparable chemical shifts and splitting patterns for H-14 and H-7α [8]. The presence of both groups mentioned above was confirmed by NOE correlations and $^{1}H-^{1}H$ COSY experiment [9,10]. In the downfield region of the ^{1}H NMR spectrum, signals appeared at δ 5.82 (1H, dd, $J = 10.5$, 17.5 Hz) and δ 4.95 (1H, dd, $J = 1.9$, 10.5 Hz) attributable to H-15 and H-16 indicating the presence of a double bond at C-15. Structural confirmation and specific assignments of the protons in **1**, in particular those of the ethylenic group, were provided by a combination of COSY and HMBC data. Specifically, the HMBC spectrum displayed long-range correlations from the H-14 and C-15, C-16, and C-17 (Figure 1).

A methyl malonate substitution was evidenced by the signals at δ 169.7, 167.2, 50.3, and 47.1 in the C NMR spectrum together with those at δ 3.41 (s, 2H) and 3.61 (s, 3H) in the ^{1}H NMR spectra. The location of methylmalonyloxy group in this compound was established from HMBC data (Figure 2), which displayed long-range correlations from the H-3β proton (δ 1.52) to the signals of carbonyl malonate (δ 167.2) and C-18 methylene protons (δ 1.24). COSY data revealed cross-peaks for Me-20, H-11β, H-12β, and Me-17 suggesting that all these protons were on the same face of the molecule (β). The H-9 showed correlations with H-5α and with H-11α and H-12α, implicating a *syn*-relationship among these protons (Figure 2). The structure of **1** was thus established as 7β-hydroxy-19α-methyl-malonyloxy-isopimara-8(14),15-diene.

Compound **2** was obtained from the same fraction as compound **1**. The spectroscopic properties of **2** were similar to those of **1**. IR absorption bands indicated the presence of the same functional groups as in **1**. The molecular formula was established by HR-EI-MS to be $C_{21}H_{30}O_4$ (ion peak at *m/z* 346.2163). Long-range $^{1}H-^{13}C$ correlations between the protons at δ 5.77 (H-15) and δ 1.14 (Me-17) and the ketonic carbon at δ 201.3 were supported by ketonic group at C-14; in addition, chemical shift value was in agreement with conjugation with the double bond at C-8 and C-9. The HMBC correlation of H_3-20 to C-9 also supports evidence for the double bond between C-8 and C-9. Furthermore, the UV spectrum of

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1**–**3** (δ in ppm).

	1		**2**		**3**	
Position	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1α	36.81	1.76 (dd, 8.7,1.5)	39.34	1.74 (dd, 8.7, 1.4)	38.27	1.79 (dd, 8.7, 1.5)
1β		1.10 (dd, 8.7, 4.3)		1.03 (dd, 8.7, 4.1)		1.30 (dd, 8.7, 4.3)
2α	19.12	1.61, m	18.51	1.54 (dt, 14.3, 1.4)	18.70	1.68, m
2β		1.10 overlapped		1.10 overlapped		
3α	36.21	1.96 overlapped				
3β	–	1.52 overlapped	37.10	1.64 (dd,14.3, 1.4)	36.52	1.69 (dd,14.6, 1.5)
4	37.84	–	36.80	–	37.66	–
5	53.23	1.50 (dd, 12.6, 1.8)	50.37	2.38,m	28.98	1.53 (dd,14.6, 1.5)
6	29.72	1.70, m	28.61	1.64, m	29.63	1.75, m
7α	70.46	4.62 (t, 1.5)	72.03	4.61 (dd,5,1.5)	71.30	4.64 (t, 1.5)
8	138.91	–	125.25	–	51.32	1.84, m
9α	39.62	2.71 (td, 7.0, 2.0)	138.37	–	138.29	–
10	53.27	–	35.91	–	38.975	–
11	20.83	1.82, m	20.82	1.86, m	128.77	5.42 br, s,
12	35.11	1.44, m	36.26	1.44, m	37.18	2.34 (m, 12a) 2.14 (m, 12b)
13	38.25	–	53.54	–	38.31	–
14	136.54	5.60 (d, 2.0)	201.37	–	203.59	–
15	142.61	5.82 (dd, 10.5, 17.5)	147.1	5.77 (dd, 10.5, 17.5)	142.46	5.80 (dd,10.5, 17.5)
16	115.38	4.95 (dd, 1.9, 10.5) 5.10 (dd, 1.9, 17.5)	113.73	5.12 (dd, 1.9, 17.5)	115.04	4.94 (dd, 1.9, 10.5) 5.11 (dd, 1.9, 17.5)
17	27.90	1.12, s	26.82	1.14, s	26.93	1.12, s
18	28.45	1.24, s	26.93	1.22, s	27.47	1.26, s
19	68.94	4.42 (d, 10.9)	65.69	4.40 (d,10.9)	68.80	4.43 (d, 10.9)
20	17.90	0.87, s	17.64	0.89, s	18.65	0.91, s
$\underline{CO_2}CH_2–CO_2CH_3$	167.26	–	–	–	167.48	–
$\overline{CO_2}\underline{CH_2}–CO_2CH_3$	47.18	3.41, s	–	–	47.52	3.44, s
$CO_2\overline{CH_2}–\underline{CO_2}CH_3$	169.73	–	–	–	169.53	–
$CO_2CH_2–\overline{CO_2}\underline{CH_3}$	50.33	3.61, s	–	–	50.84	3.63, s
CO_2H	–	–	177.18	11.06, s	–	–

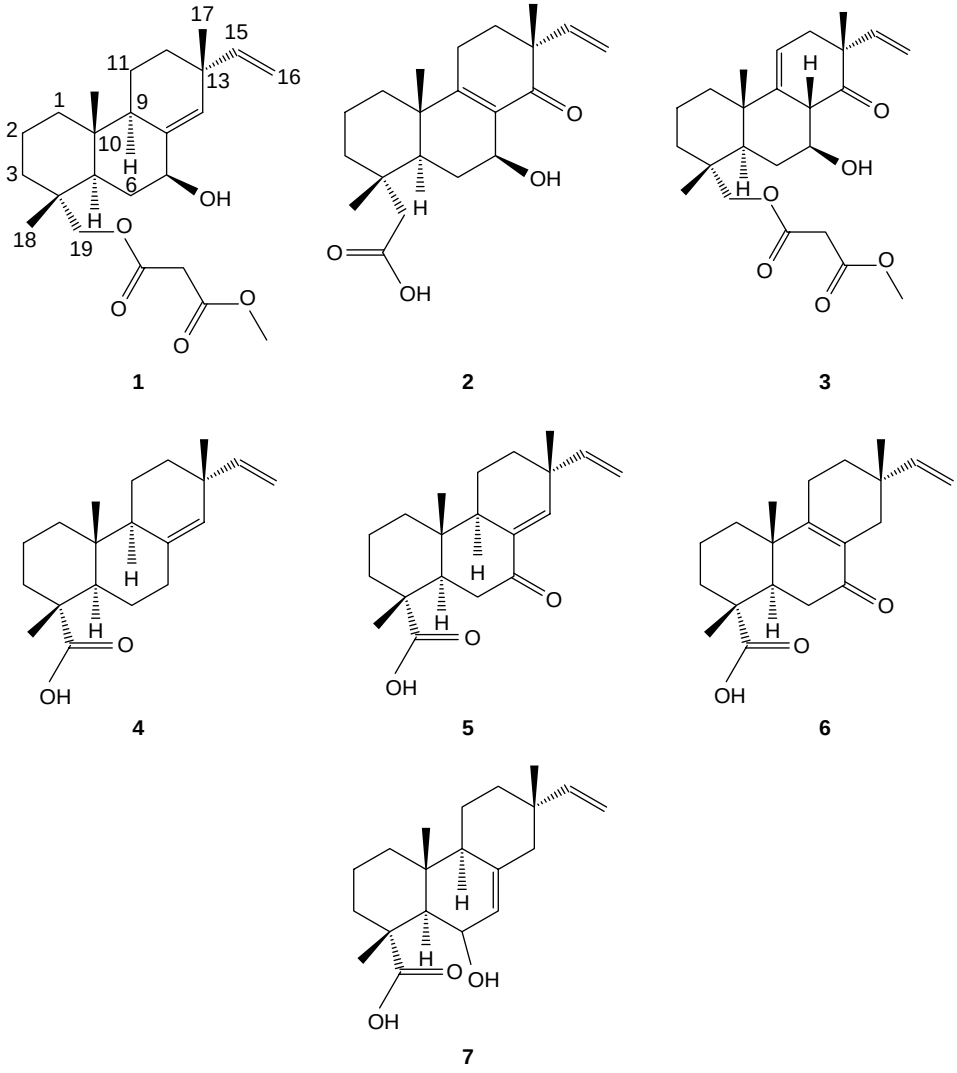

Figure 1. The structures of compounds **1**–**7**.

2 which exhibited an absorption band at 262 nm corroborate the presence of an α,β-unsaturated ketone. However, a significant shift for C-19 compared with **1** indicated substitution at this position. Also consistent with these observations was a carboxyl substitution at this position, as evidenced by the signals at δ 11.0 and δ 177.1 [11]. On the basis of HMBC correlations, we assigned **2** as 7β-hydroxy-14-oxo-isopimara-8(9),15-dien-19-oic acid.

The ^{1}H and ^{13}C NMR spectra of compound **3** were very similar to those of **1** as both compounds possessed the same isopimara-15-en skeleton. In compound **3**, the presence of a carbonyl group was also observed at C-14 like **2**, according to the HMBC correlations between the H-15, H-17 with a ketonic carbon at δ 203.5. Finally, HMBC correlations between H-12 C-8, C-9, and C-11 and between H-11, C-8, C-12, C-13, and C-14 confirmed the C-9/C-11 double bond. Consequently,

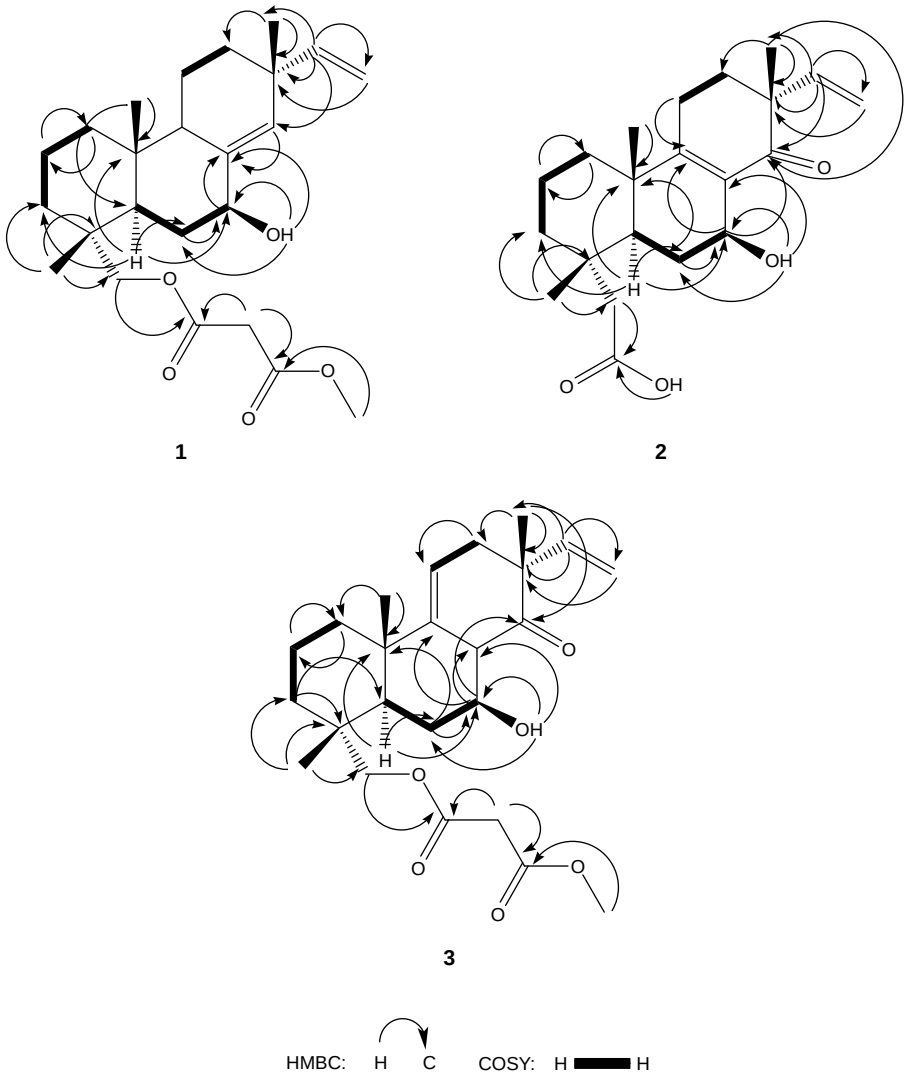

Figure 2. Selected HMBC and COSY correlations of **1–3**.

the structure of **3** was elucidated as 7β-hydroxy-14-oxo-19α-methylmalonyloxy-isopimara-9(11),15-diene.

The crude extract of hexane of *R. hieroglyphicum* examined by diffusion method revealed inhibition against microbial growth having zone of inhibition at 250 μg/ml. The extract was systematically divided by chromatography, the fractions showed significant antimicrobial growth having zone of inhibition ranging from 19.56 to 24.76 mm. The results show that the fractions have components responsible for the antimicrobial effects observed in *in vitro* analysis; special attention was focused on isolating the active principle from each fraction. The obtained compounds were tested for their antimicrobial activity by disk diffusion method by measuring the zone of inhibitions, and for the most active compounds, minimum inhibitory concentration (MIC) values were also determined. These activities are comparable with the effects of

Table 2. Antibacterial activity of compounds **1**–**7** isolated from *R. hieroglyphicum* using agar disk diffusion and MIC methods.

	MIC μg/ml								
	1	2	3	4	5	6	7	8	9
B. subtilis	13	9	12	11	7	8	10	20	–
S. epidermidis	8	6	7	8	5	5	7	10	–
S. aureus	16	12	14	13	10	11	14	13	–
E. coli	17	12	15	14	9	10	15	19	–
K. pneumonia	12	7	11	10	5	6	9	19	–
P. mirabilis	14	11	15	13	7	9	12	19	–
P. aeruginosa	18	11	17	16	8	10	13	16	–
C. albicans	17	14	17	18	10	12	17	–	21
C. tropicalis	19	13	18	15	10	11	14	–	22
C. glabrata	20	16	19	18	12	13	18	–	21

Notes: 8, netilmicin; 9, intraconazole.

netilmicin for bacteria and intraconazole for fungi. The results showed interesting and promising antimicrobial activity (Table 2). Compounds **5** and **6** showed better inhibitory activity against the microorganisms (MIC values: 5–13 μg/ml), whereas compounds **1**, **2**, **3**, **4**, and **7** also exhibited very strong antimicrobial as well as broad spectrum against both fungi and bacteria (MIC values: 6–20 μg/ml).

In conclusion, the results showed that algae, *R. hieroglyphicum,* displayed a good activity against gram-positive and gram-negative bacteria and fungal strains studied, indicating that isopimarane diterpenes are important metabolites in the search for new effective antibacterial agents against pathogens. Hexane extract and the isolated chemical compounds have shown antimicrobial activity, confirming the traditional reputation of xochitl as an antimicrobial agent.

3. Experimental

3.1 General experimental procedures

IR spectra were recorded on a Perkin-Elmer FT-IR 1720X. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker DRX-300 NMR spectrometer, with UXNMR software package, which was used for NMR experiments; chemical shifts are reported in δ (ppm), downfield relative to TMS as an internal standard. The NMR experiments were carried out using the conventional pulse sequences as described in the literature. HR-EI-MS were measured on a JEOL HX 110 mass spectrometer. The apparatus used for $[\alpha]_D$ was Strainoptics PS-100 Polarimeter. The apparatus used for UV data was Beckman Coulter DU 800 UV/vis. Precoated TLC silica gel 60 F254 aluminum sheets and Sephadex LH-20 from Sigma-Aldrich (St Louis, MO, USA) were used. Column chromatography was carried on Silica gel 60 (230–400 mesh; Merck Co., Whitehouse Station, NJ, USA; solvents used as eluents were obtained from Fermont (CA, USA).

3.2 Source sample collection

R. hieroglyphicum belongs to the family of Cladophoraceae. Field samples were collected in April 2009 in Balsas basin, in south-central Mexico and were taxonomically authenticated at the Phycology Laboratory, Faculty of Sciences, UNAM and a voucher specimen of the algae is stored for reference (6879). After the collection, the samples were kept in an ice chest. Upon return to the laboratory, the samples were washed with distilled water and extraneous plant/animal material was removed.

3.3 Extraction and isolation

Dried and ground materials (1.5 kg) were refluxed with hexane (12 liters) two times successively, for 3 h. The hexane extracts were combined and evaporated under reduced pressure to give a dry residue (50 g). Fractionation was monitored by TLC with visualization under UV (254 and 365 nm). Fractions of 20 ml each were collected. The residue was subjected to column chromatography (silica gel, Merck Co) eluting with $CHCl_3$–hexane 12:1 producing six pooled fractions F1–F6, which were tested for their antimicrobial activity. F2 (14.5 g) was the fraction that showed antimicrobial properties. Fraction F2 was chromatographed on a silica gel column using as gradient $CHCl_3$—EtOAc (12 → 3) to yield five secondary fractions (F2-1 to F2-5). The most active fractions were F2-1 (1.1 g), F2-3 (5.3 g), and F2-5 (6.1 g) in that order. F2-3 was subjected to column chromatography on silica gel eluted with a mixture of hexane–acetone–ethyl ether (3:1.5:0.5), affording five fractions F2-3-1 to F2-3-5. Fractions F2-3-3 (2.8 g) and F2-3-5 (1.7 g) showed antimicrobial activity, which were individually subjected to further column chromatography eluting with hexane–$CHCl_3$–EtOAc (10:1:1) to obtain the subfractions F2-3-3-1 to F2-3-3-4 and F2-3-5-1 to F2-3-5-6, respectively. The active subfractions F2-3-3-2 and F2-3-5-4 were subjected to repeated column chromatography on Sephadex LH-20 (chloroform with increasing amounts of methanol) to obtain **1** (38 mg), **2** (39 mg), **3** (45 mg), and **4** (37 mg). F2-5 was subjected to column chromatography over silica gel and eluted with a gradient of hexane–acetone–ethyl ether (4:1.5:1.0) to produce seven fractions (F2-5-1 to F2-5-7). The secondary subfraction F2-5-3 (4.3 g) was subjected to repeated column chromatography, first on silica gel (chloroform–acetone, 7:0.5) and then on Sephadex LH-20 to obtain **5** (29 mg), **6** (32 mg), and **7** (49 mg).

3.3.1 7β-Hydroxy-19α-methylmalonyloxy-isopimara-8(14),15-diene (*1*)

A colorless oil; $[\alpha]_D^{20}$ + 38.4; IR ($CHCl_3$): ν_{max} 3400 (OH), 2918, 2844, 1750 (carbonyl ester), 1699 (C=C), 1100, 956, 804 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), spectral data are given in Table 1. HR-EI-MS *m/z* 404.2575 $[M + H]^+$ (calcd for $C_{24}H_{36}O_5$, 404.2563).

3.3.2 7β-Hydroxy-14-oxo-isopimara-8(9),15-dien-19-oic acid (*2*)

A colorless oil; $[\alpha]_D^{20}$ + 57.2; UV ($CHCl_3$) λ_{max} 262 nm; IR ($CHCl_3$) ν_{max} 3075, 3401 (OH), 1732 (COOH), 1668, 1660 (α,β-unsaturated carbonyl), 3074, 985, 905 (vinyl group) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), spectral data are given in Table 1. HR-EI-MS *m/z* 346.2163 $[M + H]^+$ (calcd for $C_{21}H_{30}O_4$, 346.2144).

3.3.3 7β-Hydroxy-14-oxo-19α-methylmalonyloxy-isopimara-9(11),15-diene (*3*)

A colorless oil, $[\alpha]_D^{20}$ + 47.6; IR ($CHCl_3$) ν_{max} 3420 (OH), 2920, 2847, 1752 (carbonyl ester), 1690 (C=C) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), spectral data are given in Table 1. HR-EI-MS *m/z* 418.2341 $[M + H]^+$ (calcd for $C_{24}H_{34}O_6$, 418.2356).

3.4 Antimicrobial screening test

The twofold dilution cup-plate agar diffusion method [10] was used to determine the MIC of all the derivatives. A known concentration of each derivative or the standard antibiotic (ampicillin, 10 μg/ml) was prepared in 10% (v/v) DMSO. One milliliter of a standardized suspension (10^6 cells/ml) of each test micro-organism was seeded into 20 ml of Mueller-Hinton agar (MHA) in sterile Petri dishes and allowed to solidify on a flat surface. Using

a sterile 6 mm corkborer, we bored six holes on each MHA plate and introduced the various serially diluted drug solutions (40 μl) into the holes. The plates were incubated at 37°C for 24 h. The resulting inhibition zone diameter (IZD) was measured after the incubation period. A plot of logarithm of drug concentration against IZD^2 afforded the estimation of the MIC of the drugs according to a reported method [4]. The experiments were carried in duplicate.

3.5 Antimicrobial activity

Ten micro-organisms were assayed which were three gram-positive bacteria *Staphylococcus aureus* (ATCC6538), *Staphylococcus epidermidis* (ATCC12228), and *Bacillus subtilis* (PCI 219), four gram-negative bacteria *Escherichia coli* (HB101), *Klebsiella pneumoniae* (ATCC4352), *Pseudomonas aeruginosa* (ATCC 227885), and *Proteus mirabilis* (ATCC14153) as well as the pathogenic fungi *Candida albicans* (ATCC10231), *Candida glabrata* (ATCC28838), and *Candida tropicalis* (ATCC13801), obtained from the microbiology laboratory of the Escuela Nacional de Ciencias Biologicas (IPN).

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