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Antimicrobial sesquiterpenoids from *Laurencia obtusa* Lamouroux

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Abstract: Purification of the organic extract of *Laurencia obtusa* Lamouroux by column chromatography and preparative thin layer chromatography provided four new compounds: a eudesmane-type sesquiterpenoid [eudesma-4(15),11-diene-5,7-diol (**1**)], a cuparane-type sesquiterpenoid [10-hydroxycuparaldehyde (**2**)], and two *nor*-cuparanes [3-hydroxy-15-*nor*-cuparan-10β-ol (**3**) and 2-bromo-3-hydroxy-15-*nor*-cuparan-10β-ol (**4**)]. Structural identification was made possible by comparison of spectral data with those reported in the literature. Compounds **3** and **4** are significant as *nor*-cuparanes are rarely isolated from marine environment. **1** showed moderate anticandidal activity, whereas **2** exhibited reasonable antibacterial activity against multidrug-resistant bacteria (especially Gram-positive). All the compounds are nontoxic to *Artemia salina*.

Keywords: Terpenoids, antimicrobial, red algae, Red Sea

1 Introduction

Laurencia is a common genus of marine red algae, taxonomically classified as Rhodophyta, Rhodophyceae, Ceramiales, Rhodomelaceae. *Laurencia obtusa* is one of the most widely investigated marine species by natural product chemists. It provides a unique source of halogenated secondary metabolites such as C_{15}-acetogenines, diterpenes, and sesquiterpenoid skeletons [1-9]. Many of these compounds are biologically active, showing antioxidant, antimalarial, antimicrobial, and cytotoxic activity [3,6,10].

To find new bioactive compounds in marine macro-organisms, *Laurencia obtusa* Lamouroux was collected from the Saudi Red Sea and its organic fraction extracted to provide four new sesquiterpenoids (Figure 1). The antimicrobial and anticandidal activities of the new compounds were also investigated.

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2 Experimental

2.1 General

Column chromatography was performed with aluminum oxide Fluka, neutral type 507C. Fractions were analyzed by TLC on silica gel 60 F254. Preparative TLC was performed on glass plates (20 cm x 20 cm) coated with silica gel (250μm thickness). Spots were visualized using UV light (254 nm), then *p*-anisaldehyde-sulfuric acid spray reagent. High resolution electron impact mass spectra (HREIMS) were recorded on a Krators EIMS-25 instrument at an ionizing voltage of 70 eV. 1D and 2D NMR spectra were recorded on a Bruker 850 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) using the solvent residual signal as the internal standard ($CDCl_3$: δ 7.26 for ^1H and δ 77.0 for ^{13}C).

2.2 Extraction and Isolation

Laurencia obtusa Lamouroux was collected in May 2016 from Salman Gulf, north of Jeddah, Saudi Arabia (21°51'39.8" N; 38°58'42.7" E). Reference standard (JAD 03060) was stored at the Faculty of Marine Sciences, King Abdulaziz University (Jeddah, Saudi Arabia). *L. obtusa* was dried, then extracted with equal volumes

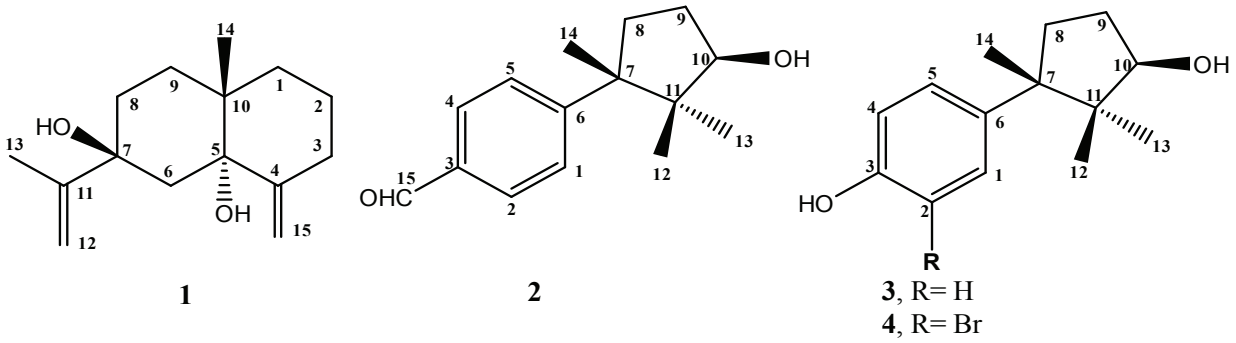

Figure 1: Structure of compounds **1–4**.

of dichloromethane and methanol. The residue (6 g) was purified first by column chromatography on Sephadex LH-20 (MeOH/$CHCl_3$ = 9.5:0.5), then by column chromatography on neutral aluminum oxide using gradient elution (*n*-hexane/diethyl ether to *n*-hexane/EtOAc). Fractions containing the product were combined and dried under reduced pressure. Final purification by PTLC afforded the pure product.

2.3 Chemical characterization

Eudesma-4(15),11-diene-5,7-diol (1); purified by column chromatography (eluent: 30% diethyl ether) followed by PTLC (eluent: 30% diethyl ether). The violet band (*p*-anisaldehyde-sulfuric acid reagent) was collected to provide **1** as a colorless liquid (1.4 mg, 0.0007% yield). R_f 0.30; $[\alpha]_D$ +54.0 (*c* 0.014, CH_2Cl_2); IR *ν* (cm^{-1}) 3424, 2924, 2854, 1712, 1640, 1455, 1377, 1261, 1172, 1074, 904; EI-MS *m*/*z* 236; HREIMS *m*/*z* 236.1764 (Calcd. 236.1776, $C_{15}H_{24}O_2$); ^1H and ^{13}C NMR (Tables 1 and 2, respectively).

10-Hydroxycuparaldehyde (**2**); purified by column chromatography (eluent: 15% diethyl ether) followed by PTLC (eluent: 10% diethyl ether). The brown band (*p*-anisaldehyde-sulfuric acid reagent) was collected to provide **2** as a pale yellow liquid (4.5 mg, 0.0023% yield). R_f 0.53; $[\alpha]_D$ +79.0 (*c* 0.023, CH_2Cl_2); UV (MeOH) λ_{max} 252, 259 and 290 nm; IR *ν* (cm^{-1}) 3274, 2972, 2927, 1701, 1606, 1461, 1392, 1369, 1223, 1178, 1075, 854; EI-MS *m*/*z* 232; HREIMS *m*/*z* 232.1451 (Calcd. 232.1463, $C_{15}H_{20}O_2$); ^1H and ^{13}C NMR (Tables 1 and 2, respectively).

3-Hydroxy-15-*nor*-cuparan-10β-ol (**3**); purified by column chromatography (eluent: 40% diethyl ether) followed by PTLC (eluent: 30% diethyl ether). The light blue band (*p*-anisaldehyde-sulfuric acid reagent) was collected to provide **3** as a colorless liquid (0.7 mg, 0.0004% yield). R_f 0.4; $[\alpha]_D$ +61.8 (*c* 0.007, CH_2Cl_2); UV (MeOH) λ_{max} 230 and 280 nm; IR *ν* (cm^{-1}) 3383, 2921, 2851, 1736, 1605, 1463, 1377, 1286, 1239, 1183, 1075, 832; EI-MS *m*/*z* 220; HREIMS *m*/*z* 220.1451 (Calcd. 220.1463, $C_{14}H_{20}O_2$); ^1H and ^{13}C NMR (Tables 1 and 2, respectively).

2-Bromo-3-hydroxy-15-*nor*-cuparan-10β-ol (**4**); purified by column chromatography (eluent: 40% diethyl ether) followed by PTLC (eluent: 30% diethyl ether). The light blue band (*p*-anisaldehyde-sulfuric acid reagent) was collected to provide **4** as a colorless liquid (0.7 mg, 0.0004% yield). R_f 0.4; $[\alpha]_D$ +50.4 (*c* 0.007, CH_2Cl_2); UV (MeOH) λ_{max} 230 and 280 nm; IR *ν* (cm^{-1}) 3383, 2921, 2851, 1736, 1605, 1463, 1377, 1286, 1239, 1183, 1075, 832, 557; HREIMS *m*/*z* 298.0556 and 300.0536 (1:1) (Calcd. 298.0568 and 300.0548 for $C_{14}H_{19}{}^{79}BrO_2$ and $C_{14}H_{19}{}^{81}BrO_2$, respectively); ^1H and ^{13}C NMR (Tables 1 and 2, respectively).

2.4 Biological evaluation

2.4.1 Antibacterial activity

Gram-negative and Gram-positive bacteria (Table 3) were obtained from urine samples of patients at the King Fahad General Hospital (Saudi Arabia) and identified according to standard guidelines [11].

The antibacterial activity was tested on Muller-Hinton agar using agar diffusion well test [12]. The minimum inhibitory concentration (MIC) was determined by ELISA using 96-well plates and fluorescein diacetate (FDA, 5 µl of a 0.25% w/w in acetone) as an indicator. The green color due to FDA hydrolysis was estimated at λ_{max} 490 nm using the ELISA tray reader [13,14].

Compound toxicity was assessed using *Artemisia salina* as test creature and dimethylsulfoxide (DMSO) as a negative control [15].

Table 1: ^{1}H NMR data of compounds **1–4** ($CDCl_3$, 850 MHz).

No.	1	2	3	4
	δ_H ppm (mult., *J*/Hz)	δ_H ppm (mult., *J*/Hz)	δ_H ppm (mult., *J*/Hz)	δ_H ppm (mult., *J*/Hz)
1	H_a 2.05 (ddd, 17.9, 13.6, 4.3) H_b 1.25-1.27 (m)	7.39 (d, 8.5)	7.07 (d, 8.5)	7.14 (d, 2.6)
2	H_a 1.99 (ddd, 17.9, 13.6, 4.3) H_b 1.55-1.57 (m)	7.79 (d, 8.5)	6.74 (d, 8.5)	-
3	H_a 2.32-2.35 (m) H_b 2.17-2.22 (m)	-	-	-
4	-	7.79 (d, 8.5)	6.74 (d, 8.5)	6.92 (d, 8.5)
5	-	7.39 (d, 8.5)	7.07 (d, 8.5)	7.02 (dd, 8.5, 2.6)
6	2.25 (d,14.5) 1.50 (dd,14.5, 2.6)	-	-	-
7	-	-	-	-
8	1.59-1.62 (m)	H_a 2.34 (ddd, 14.5, 9.4, 5.1) H_b 2.02 (ddd, 13.6, 12.8, 5.1)	H_a 2.22-2.27 (m) H_b 1.94-1.98 (m)	H_a 2.22-2.27 (m) H_b 1.94-1.98 (m)
9	H_a 1.88 (ddd, 17.9, 12.8, 5.1) H_b 1.21-1.24 (m)	H_a 2.57 (ddd, 14.5, 9.4, 6.0) H_b 2.28-2.31 (m)	H_a 2.48-2.52 (m) H_b 2.19-2.25 (m)	H_a 2.48-2.52 (m) H_b 2.19-2.25 (m)
10	-	4.01 (dd, 9.4, 9.4)	4.04 (dd, 10.2, 9.4)	4.02 (dd, 10.2, 9.4)
11	-	-	-	-
12	H_a 5.04 (s) H_b 4.83 (s)	1.13 (s)	1.08 (s)	1.08 (s)
13	1.82 (s)	0.62 (s)	0.61 (s)	0.63 (s)
14	0.93 (s)	1.48 (s)	1.41 (s)	1.40 (s)
15	H_a 5.06 (s) H_b 4.84 (s)	9.99 (s)	-	-

Table 2: ^{13}C NMR data of compounds **1–4** ($CDCl_3$, 212 MHz).

No.	1	2	3	4
1	31.6, CH_2	128.2, CH	128.7, CH	127.9, CH
2	31.2, CH_2	128.9, CH	114.2, CH	119.1, C
3	33.3, CH_2	134.4, C	153.6, C	149.4, C
4	151.6, C	128.9, CH	114.2, CH	115.1, CH
5	78.3, C	128.2, CH	128.7, CH	127.6, CH
6	40.6, CH_2	154.5, C	139.3, C	140.6, C
7	75.0, C	48.8, C	47.6, C	47.6, C
8	22.3, CH_2	36.5, CH_2	36.6, CH_2	36.5, CH_2
9	34.2, CH_2	33.1, CH_2	33.1, CH_2	33.0, CH_2
10	38.5, C	61.1, CH	62.0, CH	61.5, CH
11	150.9, C	49.6, C	48.3, C	48.5, C
12	109.6, CH_2	21.1, CH_3	20.9, CH_3	21.0, CH_3
13	18.9, CH_3	22.6, CH_3	22.5, CH_3	22.7, CH_3
14	22.2, CH_3	25.1, CH_3	25.2, CH_3	25.2, CH_3
15	108.2, CH_2	191.8, CH	-	-

2.4.2 Anticandidal activity

MIC was determined using the method described by Chand *et al.* [13] and modified by Aly and Gumgumji [14].

3 Results and discussion

Four new sesquiterpenoids (**1**–**4**) were isolated from *Laurencia obtusa* Lamouroux after extraction and purification of its organic fraction. Spectroscopic analysis of the four compounds afforded the following results.

Compound **1** was isolated as a liquid with specific rotation $[\alpha]_D$ +54 (*c* 0.014, CH_2Cl_2). HREIMS analysis of the molecular ion peak at *m*/*z* 236.1764 provided the molecular formula $C_{15}H_{24}O_2$, which requires four degrees of unsaturation. The lack of absorption in the UV spectrum revealed the absence of conjugation. Hydroxyl group (ν 3424 cm^{-1}) and exocyclic C=C bond (ν 1640 cm^{-1}) were assigned from the IR spectrum. The ^{13}C NMR spectrum displayed 15 signals, which were categorized by DEPT into

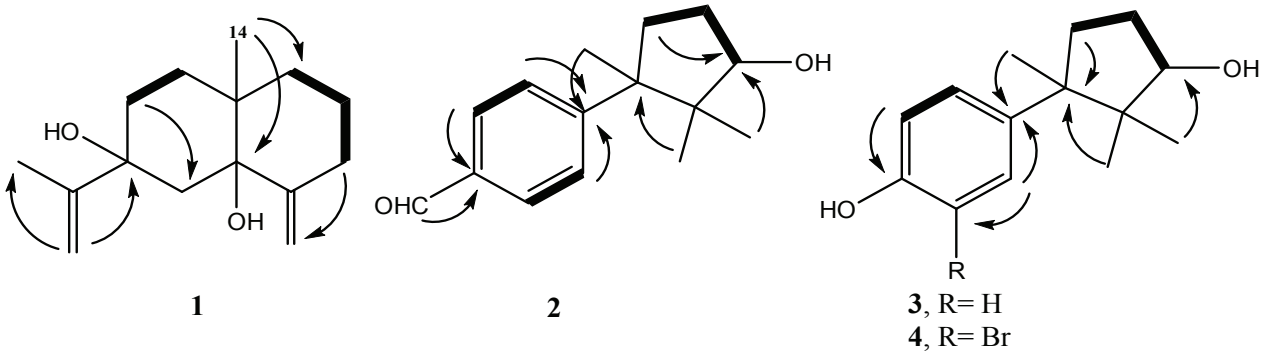

Figure 2: Selected H-H COSY (▬) and HMBC correlations of compounds **1-4** (↷).

5 quaternary, 8 methylene and 2 methyl carbons. HSQC experiment indicated the presence of two tertiary methyls (δ_H/δ_C = 1.82/18.9 and 0.93/22.2), two oxygenated quaternary carbons (δ_C 78.3 and 75.0), two olefinic quaternary carbons (δ_C 151.6 and 150.9), and two exocyclic methylene groups (δ_H/δ_C 5.06 and 4.84/108.2; δ_H/δ_C 5.04 and 4.83/109.6; Tables 1 and 2). The ^{1}H—^{1}H correlation experiment highlighted three sequences: three correlated methylene groups, two correlated methylene groups, and an isolated methylene group (δ_H 1.50 and 2.25). Overall, these results suggest that compound **1** is an eudesmane-type sesquiterpenoid with two C=C bonds fulfilling four degrees of unsaturation. HMBC experiment revealed additional correlations: the methyl protons at δ_H 0.93 (Me-14) correlate to carbons at δ_C 78.3, 34.2 and 31.6, confirming that Me-14 is an angular methyl; methyl protons at δ_H 1.82 (Me-13) correlate to carbons at δ_C 150.9, 109.6 and 75.0, confirming that Me-13 is part of the isopropylidene group (Figure 2). The relative configuration of **1** was assigned by NOESY experiment as well as by analogy with known compounds. The absence of correlation between Me-13 and Me-14 in NOESY spectrum suggests the two groups are not co-facially oriented. As in natural eudesmanes Me-14 is β-oriented,[7] Me-14 and OH group at C-5 must be *trans* correlated. Based on structure, **1** was named eudesma-4(15),11-diene-5,7-diol.

Compound **2** was isolated as a liquid with specific rotation $[\alpha]_D$ +79.2 (*c* 0.025, CH_2Cl_2). HREIMS analysis of the molecular ion peak (*m/z* 232) provided the molecular formula $C_{15}H_{20}O_2$, which requires six degrees of unsaturation. The UV absorptions at 252, 259 and 290 nm indicated a substituted benzene ring. The IR spectrum showed the characteristic bands of OH, C=O and *gem* dimethyl groups (*v* 3274, 1701 and 1369 cm^{-1}, respectively). The presence of the OH group was confirmed by a peak at *m/z* 214 [M–H_2O] in the EI-MS spectrum. The ^{13}C NMR spectrum of **2** showed 15 signals, categorized by DEPT into 4 quaternary, 6 methine, 2 methylene and 3 methyl carbons. The ^{1}H—^{13}C HSQC experiment revealed the presence of the following groups (Tables 1 and 2): three aliphatic methyl groups (singlets at δ_H/δ_C 1.48/25.1, 1.13/21.1 and 0.62/22.6), a formyl group (singlet at δ_H/δ_C 9.99/191.8), and a *p*-disubstituted benzene ring (two doublets in the aromatic region at δ_H/δ_C 7.79 [*J* = 8.5 Hz, 2H]/128.9 and δ_H 7.39[*J* = 8.5 Hz, 2H]/128.2). In addition, the proton-proton correlation spectrum revealed two proton sequences: the aromatic system and a CH_2—CH_2—CH fragment. The aforementioned analysis points to a cuparane-type sesquiterpenoid structure containing a cyclopentane ring connected to a benzaldehyde skeleton. The location of the aldehyde group in the *p*-position of the benzene ring was confirmed by HMBC correlation of the aldehyde proton (H-15, δ_H 9.99) to C-3 (δ_C 134.4), C-2 (δ_C 128.9), and C-4 (δ_C 128.9). Correlations of H-14 (δ_H 1.48) to C-7 (δ_C 48.8), C-11 (δ_C 49.6), C-8 (δ_C 36.5), and C-6 (δ_C 154.5) are also in agreement with the structure of **2**.

Comparison of the spectral data of **2** with those reported for cuparane [16] showed similarity, except for the presence of an aldehyde group in **2** in the place of a methyl group in cuparane. The increase in chemical shift value of Me-14 in compound **2** (δ_H 1.48) with respect to cuparane (δ_H 1.23) is likely explained in terms of anisotropy created by the aromatic ring as well as the electron withdrawing effect of the aldehyde group. The relative configuration of **2** was determined by NOESY experiment, which showed the correlation of Me-14 (δ_H 1.48) to Me-12 (δ_H 1.13), and of Me-13 (δ_H 0.62) to H-10 (δ_H 4.01). These correlations indicate that Me-14, Me-12 and the hydroxyl group on C-10 have the same orientation. Consequently, the relative configurations of C-7 and C-10 were assigned as 7*R** and 10*R**, respectively. Compound **2** was given the trivial name 10-hydroxycuparaldehyde.

Metabolite **3** was isolated as a liquid with specific rotation $[\alpha]_D$ +61.8 (*c* 0.007, CH_2Cl_2). Its molecular formula was determined as $C_{14}H_{20}O_2$ (with five degrees of unsaturation) by HREIMS. The UV absorptions at 230 and 280 nm indicated a substituted benzene ring. The IR spectrum revealed the presence of OH and *gem* dimethyl

groups (ν 3383 and 1377 cm^{-1}, respectively). The ^{13}C NMR spectrum of **3** showed 14 signals, categorized by DEPT into 4 quaternary, 5 methine, 2 methylene and 3 methyl carbons. The $^{1}H—^{13}C$ HSQC spectrum allowed to identify three tertiary methyls (singlets at δ_H/δ_C 1.41/25.2, 1.08/20.9 and 0.61/22.5) and a *p*-disubstituted benzene ring (two doublets in the aromatic region at δ_H/δ_C 6.74 [2H, J = 8.5 Hz]/114.2 and at δ_H/δ_C 7.07 [2H, J = 8.5 Hz]/128.7; Tables 1 and 2). Two OH groups are located on C-3 (δ_C 153.6) and C-10 (δ_H/δ_C 4.04/62.0). In addition, the proton-proton correlation spectrum revealed two proton sequences: the aromatic system and the CH_2–CH_2–CH fragment. With five degrees of unsaturation, the carbon skeleton of compound **3** must contain a (*p*-OH)C_6H_4 group attached to a 1,2,2-trimethylcyclopentan-3-ol moiety. The position of the hydroxyl groups was confirmed by HMBC correlation of the phenolic carbon C-3 (δ_C 153.6) to the aromatic protons H-2 and H-4 (δ_H 6.74), and that of H-10 (δ_H 4.04) to C-11 (δ_C 48.3) and C-9 (δ_C 33.1).

The abovementioned analysis indicates that compound **3** is a *nor*-cuparane-type sesquiterpenoid. Compound **3** was named 3-hydroxy-15-*nor*-cuparan-10β-ol.

Compound **4** (isolated as a liquid with specific rotation $[\alpha]_D$ +50.4 [*c* 0.007, CH_2Cl_2]) was identified by comparison with the spectroscopic data of **3** (Tables 1 and 2). Their IR, UV and NMR spectra were similar, with a difference in the aromatic region of the ^{1}H NMR spectrum. The $^{1}H—^{13}C$ HSQC spectrum of **4** exhibited three methine carbons in the aromatic region (δ_H/δ_C 7.14 [d, J = 2.6 Hz]/127.9, 6.92 [d, J = 8.5 Hz]/115.1 and 7.02 [dd, J = 8.5 Hz, J = 2.6 Hz]/127.6) and three quaternary carbons (δ_C 140.6, 119.1, and 149.4) indicating a 1,2,4-trisubstituted benzene ring. The molecular formula of **4** was assigned as $C_{14}H_{19}BrO_2$ by HREIMS. The parent peaks at m/z 298 and 300 in the EI-MS spectrum, with relative intensities in the 1:1 ratio, alongside the absorption band at ν 557 cm^{-1} in the IR spectrum, clearly indicate the presence of a bromine atom.

The analysis indicates that compound **4** is a *nor*-cuparane bearing two hydroxyl groups and a bromine atom. The position of bromine was assigned to C-2 (δ_C 119.1). **4** was named 2-bromo-3-hydroxy-15-*nor*-cuparan-10β-ol.

The antibacterial activity of **1** and **2** was tested against several strains of multidrug-resistant bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus aureus*). **1** displayed weak activity against all tested bacteria (results not shown), whereas **2** showed an inhibition zone of 10.0-15.5 mm diameter on Muller-Hinton agar (Table 3).

The MICs of **2** were in the range of 0.08–0.15 mM for all tested bacteria. Noteworthy, **2** was more active against Gram-positive bacteria (Table 3). All compounds showed no toxicity (LD_{50} >0.5 mM) against *Artemia salina* as test creature.

Table 3: Antimicrobial activity and minimal inhibitory concentrations (MICs) of **2** and amoxicillin on multidrug-resistant bacteria.

Bacterium[a]	Antimicrobial activity (mm)[b]		MIC (mM)	
	2	amoxicillin[c]	**2**	amoxicillin
E. coli	12.3±2.33	17.0±2.13	0.15	0.01
K. pneumoniae	10.0±2.00	22.0±4.22	0.11	0.002
P. mirabilis	14.0±3.70	24.0±1.34	0.09	0.01
P. aeruginosa	13.0±3.12	17.0±2.21	0.12	0.02
E. faecalis	15.5±2.57	19.0±1.49	0.08	0.04
S. aureus	15.0±2.56	17.0±1.09	0.09	0.08

[a] *E. coli, K. pneumonia, P. mirabilis* and *P. aeuroginosa* are Gram-negative bacteria; *E. faecalis* and *S. aureus* are Gram-positive bacteria. [b] Diameter of inhibition zone. [c] Positive control; mean ± SD (n = 3).

Compound **1** displayed anticandidal activity against *Candida albicans* and *Candida tropicalis* (MICs = 8.27 and 10.13 μM, respectively. This activity is relatively high if compared to amphotericin B (positive control; MICs = 4.63 and 5.27 μM, respectively).

4 Conclusions

Four new compounds were isolated from the organic extract of the red alga *L. obtusa* Lamouroux. These compounds belong to two major classes of sesquiterpenoids: eudesmanes [eudesma-4(15),11-diene-5,7-diol (**1**)] and cuparanes [10-hydroxycuparaldehyde (**2**), 3-hydroxy-15-*nor*-cuparan-10β-ol (**3**), and 2-bromo-3-hydroxy-15-*nor*-cuparan-10β-ol (**4**)]. Compounds **1** and **2** displayed relatively high antimicrobial activities, whereas **3** and **4** belong to a specific group of compounds (*nor*-cuparane sesquiterpenoids) that are rarely isolated from the marine environment.

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Conflict of interest: Authors declare no conflict of interest.

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