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Ursane and tirucallane-type triterpenes of *Boswellia rivae* oleo-gum resin

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ABSTRACT
Two new ursane-type triterpenes characterized as 3-oxo-24-acetoxy-11α-hydroxy-urs-12-ene (**1**) and methyl 3α-acetoxy-11α-methoxy-urs-12-en-24-oate (**2**), together with known compounds **3–11,** were isolated from *Boswellia rivae* oleo-gum resin exudate. Their structural elucidation was accomplished using physical, chemical, and spectroscopic methods. The compounds exhibited weak to moderate antibacterial activities against some Gram-positive and Gram-negative bacteria.

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Boswellia rivae; Burseraceae; terpenoids; antibacterial activities

1. Introduction

Boswellia rivae Engl. (syn: *B. boranensis* Engl.) (Burseraceae) is one of the three *Boswellia* species acclimatized in Kenya and is widely spread in the semi-arid and arid lands [1]. The species exudate a forest product, an oleo-gum resin which is used for the management of asthma, pathogenic microbes, rheumatoid arthritis, topic dermatitis, inflammatory related diseases, and chronic headache and tick repellent [2–4]. Previous phytochemical communication on this plant oleo-gum resin resulted in the isolation and identification of monoterpenes [3], diterpenes [5], triterpenes, and polysaccharides [6–8]. As part of continuing investigation for terpenoids from the oleo-gum resin exudates from Kenyan grown *Boswellia* species, we now report the isolation and characterization of two new ursane-type triterpenes, namely 3-oxo-24-acetoxy-11α-hydroxy-urs-12-ene (**1**) and methyl 3α-acetoxy-11α-methoxy-urs-12-en-24-oate (**2**) (Figure 1). Known compounds isolated included 11α-methoxy-β-boswellic acid (**3**), 3-oxotirucallic acid (**4**), a mixture of α- and β-boswellic acids (**5** and **6**) [6], lupeol (**7**), mixture of α- and β-amyrins (**8** and **9**) [9], 3β-acetoxyoleanane (**10**) [10], and kaempherol (**11**) [11]. The compounds exhibited weak to moderate antibacterial activities against some Gram-positive and Gram-negative bacteria. This is the first report of compound **4** being reported from the plant resin.

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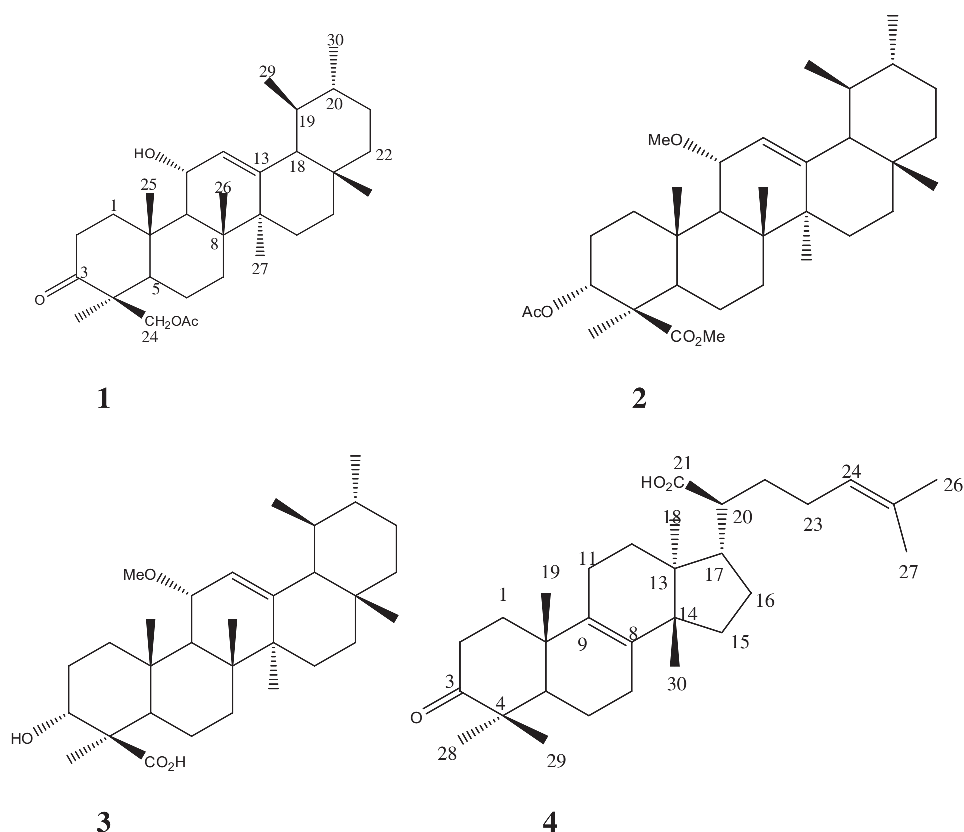

Figure 1. Compounds **1–4** isolated from *Boswellia rivae.*

2. Results and discussion

Compound **1** was obtained as white amorphous powder from 0.2% MeOH in CH_2Cl_2 and assigned a molecular formula of $C_{32}H_{50}O_4$ as evidenced by HR-ESI-MS ion peak at *m/z* 521.3607. This, together with other significant peaks at *m/z* 218 (100%) and 203(31%), suggested typical *retro*-Diels-Alder fission diagnostic of boswellic acid derivative of oleanane- or ursane-type triterpene [12,13]. The ESI-MS molecular ion peak suggested eight double bond equivalents, seven of which are assigned to the rings of the pentacyclic triterpene skeleton and the remaining was attributable to the acetoxy group (δ 169.8). An equivalent, assigned to a double bond consistent with Δ^{12}-ursane skeleton, showed resonances at δ 124.6 (C-12) and 145.2 (C-13). The IR spectrum confirmed the presence of ester C=O (1732 cm^{-1} and the C=C (1644 cm^{-1}) groups. In the ^1H NMR spectrum (Table 1) of compound **1**, the presence of vinylic proton (δ 5.36, t, J = 3.6 Hz, H-12), one oxymithine proton (δ 3.68, dd, J = 9.1, 3.5 Hz), five tertiary methyls (δ 1.31, 1.12, 1.10, 0.95, and 0.93, all singlets), and two secondary methyls (δ 0.82, *d*, J = 6.6 Hz and 0.79, *d*, J = 6.8 Hz) were observed, unambiguously confirming the presence of Δ^{12}-ursane skeleton [12]. The ^{13}C NMR spectrum (Table 2) exhibited a total of 32 carbon signals which were sorted out by 135 DEPT spectrum into nine methylenes, eight methyls, seven methines including one olefinic, and one oxygen-bearing carbons as well as eight quaternary carbons. Comparison of the ^1H and ^{13}C NMR spectra

Table 1. ^{1}H NMR spectral data of compounds **1–4** in $CDCl_3$ (600 MHz).

H	**1**	**2**	**3**	**4**
1	1.76–1.78 m, 1.10–1.12 m	1.64–1.66 m, 1.11–1.14 m	1.82–1.84 m, 1.20–1.21 m	1.96–1.98 m, 1.30–1.31 m
2	1.72–1.74 m, 1.14–1.16 m	1.68–1.69 m, 1.19–1.21 m	1. 79–1.81 m, 1.16–1.17 m	2.32–2.34 m
3		4.50 (d, *J* = 8.5, 3.2)	3.30 (dd, *J* = 8.4, 3.2)	
4				
5	1.42–1.43 m	1.40–1.41 m	1.49–1.50 m	1.36 (dd, *J* = 9.0, 5.4)
6	1.46–1.48 m	1.42–1.45 m	1.51–1.53 m	1.44–1.46 m, 1.00–1.12 m
7	1.39–1.40 m, 1.27–1.29 m	1.36–1.38 m, 1.23–1.25 m	1.34–1.35 m, 1.13–1.14 m	1.53–1.55 m, 1.35–1.37 m
8				
9	1.47 (br t, *J* = 9.3)	1.58 (t, *J* = 9.0)	1.45–1.46 m	
10				
11	3.68 (dd, *J* = 9.1, 3.5)	3.80 (d, *J* = 8.7, 2.8)	3.66 (dd, *J* = 8.8, 3.3)	
12	5.36 (t, *J* = 3.6)	5.32 (d, *J* = 3.4)	5.28 (t, *J* = 3.1)	
13				
14				
15	1.44–1.46 m	1.40–1.42 m	1.38–1.39 m	1.50–1.51 m
16	1.45–1.47 m	1.58–1.60 m	1.37–1.39 m	1.56–1.58 m
17				2.01–2.03 m
18	2.14 (d, *J* = 12.6)	2.20 (d, *J* = 10.0)	2.17 (d, *J* = 12.8)	0.82 s
19	1.55–1.56 m	1.74–1.76 m	1.57–1.58 m	0.92 s
20	1.60–1.61 m	1.57–1.59 m	1.55–1.57 m	2.37 (d, *J* = 11.5)
21	1.27–1.30 m	1.29–1.31 m	1.54–1.56 m	
22	1.42–1.44 m	1.43–1.45 m	1.45–1.48 m	
23	0.93 s	0.90 s	1.04 s	
24	3.95 (d, *J* = 11.2), 3.75 (d, *J* = 11.2)			5.10 (t, *J* = 5.7)
25	1.10 s	1.02, s	0.96 s	
26	1.12 s	1.07, s	1.10 s	1.67 s
27	1.31 s	1.23, s	1.24 s	1.60 s
28	0.95 s	0.94, s	0.89 s	0.98 s
29	0.79 (d, *J* = 6.8)	0.83 (d, *J* = 6.3)	0.81 (d, *J* = 6.5)	0.88 s
30	0.82 (d, *J* = 6.6)	0.86 (d, *J* = 6.1)	0.85 (d, *J* = 6.7)	0.89 s
CO_2		3.60, s		
Me	2.04 s	2.01 s		
OAc			3.40 s	
OMe				

Table 2. ^{13}C NMR spectral data of compounds **1–4** in $CDCl_3$ (600 MHz).

C	**1**	**2**	**3**	4
1	39.8	38.8	38.2	35.5
2	27.9	26.5	27.5	26.4
3	210.5	76.0	72.8	210.3
4	43.5	40.7	44.6	37.6
5	53.7	55.4	55.2	47.3
6	18.3	17.9	21.4	18.4
7	33.5	34.7	34.3	26.4
8	43.5	42.1	42.3	133.2
9	46.1	46.6	47.7	134.5
10	37.2	36.9	36.7	37.4
11	73.7	75.8	74.2	22.0
12	124.6	124.3	125.2	30.4
13	145.2	144.0	145.5	45.4
14	41.8	41.9	40.4	49.6
15	27.9	27.0	27.7	27.0
16	26.0	28.0	27.2	31.3
17	38.1	33.8	33.5	46.1
18	54.7	56.5	55.6	16.5
19	41.6	40.5	40.8	19.8
20	41.7	40.0	39.7	49.4
21	26.7	26.7	27.0	181.2
22	40.6	37.9	41.0	32.0
23	24.2	24.0	23.4	27.0
24	74.6	178.2	180.1	124.1
25	16.5	15.9	14.0	132.8
26	18.0	18.3	19.1	18.0
27	22.2	23.9	23.1	25.9
28	24.6	28.4	25.8	27.9
29	16.2	17.0	17.3	23.1
30	20.7	20.5	21.2	24.7
Me$\underline{CO}$	169.8	170.0		
$\underline{Me}$CO	21.6	21.4		
$\underline{CO_2Me}$		56.0		

of compound **1** with those of 11α-methoxy-β-boswellic acid (**3**) revealed close similarities with notable difference between the compounds being the substitution mode in rings A and C in **1** as substantiated by the ^{1}H and ^{13}C NMR, and ESI-MS data [*m/z* 264 ($C_{16}H_{24}O_3$) and 234 ($C_{16}H_{26}O$)]. The H-3 (*δ* 3.30, dd, *J* = 8.4, 3.2 Hz) in ring A of compound **3** was apparently replaced by a keto group with corresponding δ_c 210.5 in compound **1** confirmed by HMBC correlations (Figure 2) between the keto carbon and H-5 (*δ* 1.42–1.43 m)/ CH_3-23 (*δ* 0.93) and in turn with the exo-methylene doublets (*δ* 3.95 and 3.75, each *J* = 11.2 Hz). Also, comparing the data of compound **1** with those of synthetically derived urs-12-ene-3α, 24-diol [14] revealed a shift of the methylene peaks relatively downfield by approx. 0.70 ppm, suggesting that the exomethylene was acetylated. This was confirmed by NOESY cross peak (Figure 2) observed between the acetylated primary alcohol with CH_3-25 group [15]. Besides the acetoxy moiety, the ^{1}H NMR spectrum of **1** displayed a peak at *δ* 3.68 previously observed in boswellic acid derivatives [6,14]. The three bond long range correlations from this signal to C-8 (*δ* 43.5), C-10 (*δ* 37.2), and C-13 (*δ* 145.2) in the HMBC spectrum allowed its assignment to H-11. This proton was associated with a carbon signal at *δ* 73.7 in the HSQC spectrum and showed proton spin coupling correlation with a signal at *δ* 1.47 (H-9) and 5.36 indicating the location of hydroxyl group at C-11. The configuration of H-11 in **1** was determined to be equatorial on the basis of coupling constants with H-9 and H-12

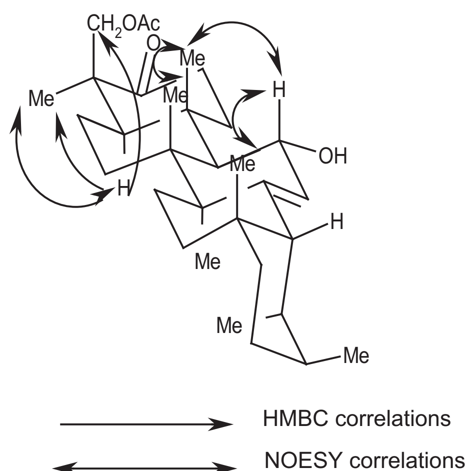

Figure 2. Significant NOESY and HMBC correlations of compound **1.**

[16] and further supported by NOESY correlation between H-11 and CH_3-25. Thus, the structure of compound **1** was established as 3-oxo-24-acetoxy-11α-hydroxy-urs-12-ene.

Compound **2** was obtained as white amorphous powder from n-hexane-CH_2Cl_2 mixture. Its IR spectrum showed significant absorption peaks attributable to acetoxy (1735 cm^{-1}), a methoxyl carbonyl (1715 cm^{-1}), and double bond (1642 cm^{-1}) functional groups. The HR-ESI-MS of the compound exhibited a pseudo-molecular ion peak at *m/z* 565. 3871 which suggested a $C_{34}H_{54}O_5$ formula containing eight unsaturation equivalents. The ^{13}C NMR spectrum (Table 2) revealed the presence of 34 carbon signals including 8 methylenes, 8 methines, 10 methyls and 8 quaternary carbons as evidenced by 135 DEPT spectrum. Signals in the low field region of the ^{13}C NMR spectrum observed at *δ* 178.2 and 170.0 confirmed the presence of the methyl carboxylic (CO_2Me) and acetoxy groups, respectively, in the molecule. The presence of an acetoxy group was further confirmed by ^{1}H NMR data (Table 1) which was compared with the known compound 3α-acetoxy-11-keto-β-boswellic acid [6]. The compound afforded signals for seven tertiary methyls (*δ* 3.60, 2.01, 1.23, 1.07, 1.02, 0.94 and 0.90, each singlet including methyls from methyl carboxylic and acetoxy groups), two secondary methyls (*δ* 0.86, d, *J* = 6.1 Hz and 0.83, d, *J* = 6.3 Hz), vinylic oxymethine (*δ* 3.80, dd, *J* = 8.7, 2.8 Hz), oxymethine (*δ* 4.50, dd, *J* = 8.5, 3.2 Hz), and an olefinic proton (*δ* 5.32, d, *J* = 3.4 Hz) in the ^{1}H NMR spectrum. These, together with the ESI-MS characteristic peaks at *m/z* 294 ($C_{17}H_{26}O_4$) and 248 ($C_{17}H_{28}O$) (Figure 3) suggested *retro*-Diels-Alder cleavage of ring C typical of ursane-type triterpene with acetoxy and methyl carboxylic groups in ring A and a methoxy moiety in ring C [17,18], an interpretation further confirming the presence of two oxygenated methine protons which correlated with carbon resonances at *δ* 76.0 (C-3) and 75.8 (C-11) in the HSQC spectrum. The position of acetoxy group on the ring system was confirmed to be at C-3 and was axially oriented as evidenced by the narrow peak half-height width $w^{1/2}$ (3.2 Hz) of equatorially positioned geminal proton which appeared relatively downfield at *δ* 4.50 [15,19,20], a fact confirmed by the HMBC correlation between H-5 (*δ* 1.40–1.41 m) and C-3 (*δ* 76.0). Similarly, the three bond long-range correlations from H-3 to carbons ascribable to C-5 (*δ* 55.4) and C-24 (*δ* 178.2) in the HMBC spectrum suggested the presence of methylated carboxylic acid group at the latter

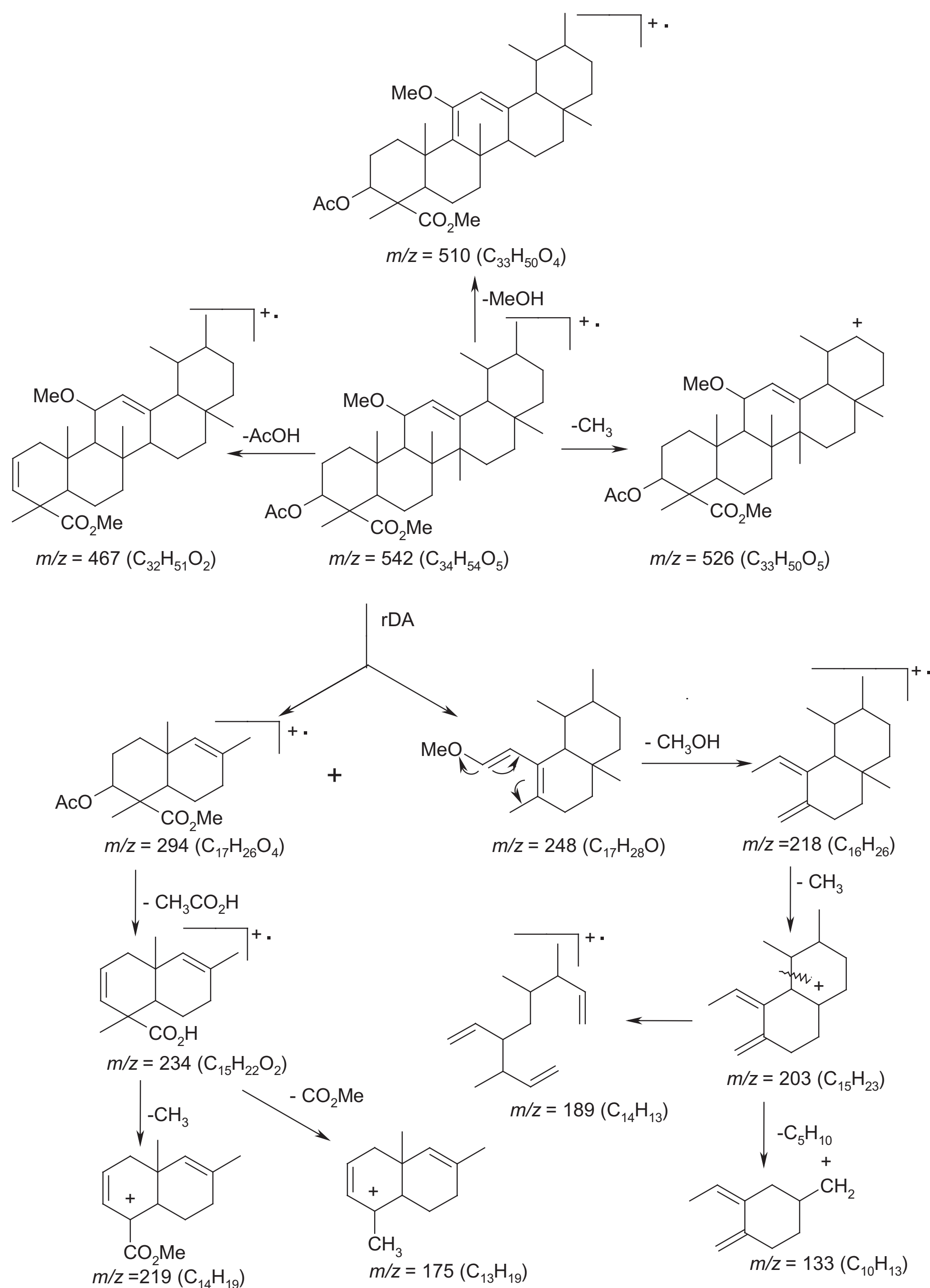

Figure 3. Possible fragmentation of compound **2** in ESI-MS.

position, possibly with β configuration, a fact confirmed by ^{1}H-^{1}H proximity (NOESY) correlation between CH_3-23 and H-5 [12]. On the other hand, a doublet at δ 5.32 (H-12) correlated with C-11 (δ 75.8)/C-8 (δ 42.1) and in turn with C-13 (δ 144.0)/C-14 (δ 41.9)

and on this basis, the position of the methoxy group was assigned to C-11; a fact further supported by ESI-MS daughter ion at *m/z* 248 ($C_{17}H_{28}O$) originating from *retro*-Diels-Alder cleavage. The configuration of the methoxyl group at C-11 was deduced to be axially orientated based on the large coupling constant (J = 8.7 Hz) which signified trans-diaxial coupling between H-11 and H-9, while small coupling constant (J = 2.8 Hz) was due to interaction of the same proton with the vinyl proton at C-12 [6], that was confirmed by the NOESY cross-peak between H-11 and H-9. Thus, on the basis of spectroscopic data as well as comparison with those in the various literature, compound **2** was deduced to be methyl 3α-acetoxy-11α-methoxy-urs-12-en-24-oate.

The CH_2Cl_2 extract displayed moderate antibacterial activity with inhibition zone diameters ranging from 14.20 mm to 26.16 mm while MeOH extract afforded values less than 10.00 mm and was considered not active (at 1.5 mg/ml). Compounds (**1–11**) were also evaluated for their antibacterial activities against Gram-positive and Gram-negative bacteria strains (Table 3). Compounds **7**, **8**, **9**, **10,** and **11** were considered inactive (MIC values > 62.5 μg/ml) when tested against the four Gram-positive and four Gram-negative bacteria. A mixture of α- and β-boswellic acids (**5** & **6**) exhibited the highest activity against *B. subtilis* with minimum inhibitory concentration (MIC) value of 7.8 μg/ml and also showed impressive activities (MIC values of 15.7 μg/ml) against *S. albus* and *S. aureus*, which is in agreement with previously reported antibacterial activity [6]. Compound **3** showed moderate activities with MIC values of 15.7 and 31.3 μg/ml against *B. subtilis*, *S. aureus* and *S. albus*, respectively. Compounds **1** and **2** also exhibited moderate activities against *B. subtilis*, *S. albus*, and *S. aureus* with MIC value of 31.3 μg/ml. From the bioassay results, interestingly it can be noted that isolated compounds were more active against Gram-positive than Gram-negative bacteria. The higher activity of α- and β-boswellic acids may be attributed to synergistic effect of the two compounds. The *in vitro* antibacterial activities results support the ethnobotanical information that *B. rivae* oleo-gum resin is a remedy of gonorrhea and other related diseases by nomadic Pokot community of Kenya [2]. Dimethylsulfoxide (DMSO) used as the negative control showed no inhibition.

3. Experimental

3.1. General experimental procedures

Optical rotation was measured on a Jasco P-1020 Polarimeter (Jasco Corporation, Tokyo, Japan). Melting points were determined on a Gallenkamp apparatus (Manchester, UK). IR data were recorded on a Bruker Tensor 27 FTIR spectrophotometer (Bruker Corporation, Bremen, Germany) as KBr pellet. The ^{1}H, ^{13}C and 2D-NMR spectra were recorded on Bruker AVANCE III-600 MHz spectrometer (Bruker Corporation, Zurich, Switzerland) equipped with a 5 mm TCI cryogenic probe head (*z*-gradient) using standard pulse sequences. Some NMR data were obtained using Bruker WM instrument operating at 250 and 62.5 MHz, respectively. TMS was used as internal standard. The mass spectral data were obtained using a Varian MAT 8200 A instrument (Bremen, Germany). Silica gel 60G (0.02–0.7 mm Mesh) Merck was used for medium pressure chromatography.

Table 3. Minimum inhibitory concentrations (μg/ml) of compounds isolated from oleo-gum resin of *Boswellia rivae* against bacteria strains.

Compounds	*E.coli*[a]	*V. cholera*[a]	*S. dysenterae*[a]	*N. meningitides*[a]	*B. subtilis*[b]	*S.albus*[b]	*D. pneumoniae*[b]	*S. aureus*[b]
1	>250	125	125	>250	31.3	31.3	125	62.5
2	>250	62.5	125	125	31.3	62.5	>250	31.3
3	125	125	62.5	62.5	15.7	31.3	62.5	15.7
4	>250	>250	125	>250	125	62.5	125	7.8
5 & 6	62.5	125	31.3	62.5	7.8	15.5	31.3	15.7
7	125	>250	>250	>250	125	>250	>250	>125
8 & 9	>250	>250	>250	>250	>125	>250	>250	>125
10	125	125	125	>250	>250	>250	>250	>250
11	>250	>250	>250	>250	>250	>250	>250	>250
Tetracycline	0.97	0.97	0.97	3.4	0.5	1.95	3.9	2.0
Gentamycin	7.8	3.9	3.9	15.7	3.9	7.8	3.9	1.95

[a]Gram-positve bacteria.
[b]Gram-negative bacteria.

3.2. Plant materials

The resin exudates were sampled in Garisa County, Kenya from authentic trees by Mr. Norman Gachathi (Taxonomist) of Kenya Forestry Research Institute (KEFRI). Voucher specimen (No: KEFRI/BR-08/2009) was deposited at the herbarium of Non-Wood Forest Products Division of the same institution.

3.3. Extraction and isolation

The gum exudate (2 kg) was extracted sequentially with CH_2Cl_2 (3 x 4 L) and MeOH (3 x 4 L), each extraction lasting four days at room temperature. Both extracts were separately evaporated under reduced pressure to afford yellow-brown oily paste (50 g) and dark-brown paste (180 g), respectively.

Approximately 45 g of the CH_2Cl_2 extract was introduced onto silica gel packed column (4.0 x 60 cm, SiO_2 500 g, pressure≈1 bar) starting with n-hexane (1.5 L), n-hexane-CH_2Cl_2 gradient (increment 10%) up to 100% of the latter and elution concluded with ethyl acetate. A total of 344 fractions were sampled with each being 20 ml. Their homogeneity was monitored by TLC using n-hexane alone, n-hexane-CH_2Cl_2 (95:5, 9:1, 4:1, and 2:1) and n-hexane-EtOAc (2:1 and 1:1), and those exhibiting similar TLC profiles were combined to constitute 5 major fractions (1–5).

Fraction 1, an oil eluted in a yield of 4 ml, was stored under freezing condition for future GC-MS analysis. Fraction 2 (eluants: 105–180, 4 g) showed three major spots R_f 0.51, 0.35, and 0.26 and were further purified into individual components using silica gel column (120 g, SiO_2), eluent: n-hexane-CH_2Cl_2 (9:1), followed by the same solvent in the ratio 4:1, to give 3β-acetoxyoleanane (1**0**, 57 mg), a mixture of α- and β-amyrin (**9** and **8**, 140 mg). Fraction 3 (eluants: 186–240, 5 g) was flash chromatographed using n-hexane-CH_2Cl_2 (7:3) followed by same solvent system in the ratio 2:1, to afford lupeol (**7,** 32 mg) and compound **1** (24 mg). Eluants 243–290 (3.6 g) constituted fraction 4 and was further purified (SiO_2, 120 g, 2.5 x 60 cm, pressure ≈1.0 bar) using n-hexane-CH_2Cl_2 (2:1) followed by the same solvent system in the ratio 1:1 to give a mixture of α- and β-boswellic acids (**5** and **6**, 100 mg), compound **3** (27 mg), and 3-oxotirucallic acid (**4,** 34 mg). Fraction 5 (eluants 295–340, 5 g) was subjected to further purification on silica gel column (SiO_2, 150 g, 3 x 50 cm) using CH_2Cl_2-n-hexane (1:1; 2:1) to afford more of **4** (21 mg) and compound **2** (35 mg).

Similarly, a portion of the methanol extract (75 g) was adsorbed onto a minimum amount of silica gel in MeOH (20 ml), solvent removed and the free flowing solid loaded onto CH_2Cl_2 silica gel packed column. Elution started with 100% CH_2Cl_2 (1.5 L) followed by CH_2Cl_2-MeOH gradient (increment of 5%) and concluded with 100% MeOH to give a total of 150 fractions (each 50 ml). Their homogeneity was monitored by TLC using CH_2Cl_2-MeOH (97:3, 95:5, 9:1 and 4:1) and the fractions were grouped into two pools (I and II) depending on their TLC profiles. Pool I (fractions 10–70, 7.5 g) on repeated medium pressure chromatography separation using CH_2Cl_2-MeOH (98:2) afforded more of **3** (15 mg) and **4** (25 mg). Pool II (fractions 80–144, 5 g) was similarly purified as in the case of pool I above using CH_2Cl_2-MeOH (95:5) followed by same solvent system in the ratio 9:1 to give compound **11** (85 mg).

3.3.1. Compound 1

White powder from CH_2Cl_2-MeOH (98:2); Mp 195–197 °C; $[\alpha]_D^{25}$ + 8.7 (c 0.5, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}: 3350, 2946, 2870, 1732, 1705, 1644, 1370, 1210, 1110, 1025, 898; ^1H and ^{13}C NMR ($CDCl_3$) spectral data: see Tables 1 and 2; ESI-MS (rel. int.): *m/z* 498 $[M]^+$ (5), 264 (14), 234 (14), 218 (100), 203 (31), 189 (24), 133 (17), 55 (70), 43 (60). HR-ESI-MS: *m/z* 521.3607 $[M + Na]^+$ (calcd for $C_{32}H_{50}O_4Na$, 521.3609).

3.3.2. Compound 2

White-amorphous powder; Mp 172–175 °C; $[\alpha]_D^{25}$ + 45 (c 0.5, $CHCl_3$); IR ν_{max} (KBr) cm^{-1}: 2930, 2870, 2500, 1735, 1715, 1642, 1460, 1250, 1110, 1020, 960; ^1H and ^{13}C NMR ($CDCl_3$) spectral data: see Tables 1 and 2; ESI-MS (rel. int.): *m/z* 543 $[M + H]^+$ (1), 542 (4), 526 (12), 510 (20), 467 (6), 294 (13), 248 (24), 234 (10), 219 (14), 218 (56), 203 (23), 189 (100), 133 (44). HR-ESI-MS: *m/z* 565. 3871 $[M + Na]^+$ (calcd for $C_{34}H_{54}O_5Na$, 565.3869).

3.4. In vitro antibacterial activity

Four Gram-positive (*Staphylococcus aureus* ATCC 25923, *Bacilus subtilis, Diplococcus pneumonia* and *Staphylococcus albus*) and four Gram-negative (*Eschirichia coli* ATCC 25922, *Vibrio cholerae, Shigella dysenterae and Neisseria meningitides*) were used for antibacterial tests. The microbes *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 were from the stock at the Microbiology Section of the Jaramogi Oginga Odinga Teaching and Referral Hospital in Kisumu County, Kenya. The rest of the organisms were clinical isolates from the same hospital. The extracts and compounds were evaluated for their *in vitro* antibacterial activities as per the National Committee for Clinical Laboratory Standards micro dilution assay format [21]. The bacteria strains were grown in prescribed media until exponential growth was achieved. The tests were performed in a 96-well micro-titer plate in a final volume of 100 μl. The test compounds were dissolved in 5% DMSO at an initial concentration of 1 mg/ml and serially diluted in the plate (with BHI broth, Becton Dickinson, Baltimore, USA) to provide decreasing concentrations. Maximum tested concentration was 1000 μg/ml while minimum tested concentration reached 1.95 μg/ml. Each well was then inoculated with 2–5 x 10^5 bacterial cells and incubated at 37 °C for 24 h. One well containing the bacteria and 5% DMSO without test compounds was for control while the other containing only growth medium was used for sterility control. Tetracycline and gentamycin were used as positive controls. The MIC was evaluated as the lowest concentration of the test substances that inhibited the growth of the bacteria strains. The assays were done in triplicate and analyzed statistically using MSTAT-C statistical package.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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