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Plastotoma rotundifolium aerial tissue extract has antibacterial activities

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ABSTRACT

Despite efforts in recent years, infectious diseases remain a worldwide major public health problem. Several infectious agents have become resistant to conventional antibiotics and there is thus urgent need to discover new antimicrobial drugs to overcome resistances. *Platostoma rotundifolium* (Briq.) A. J. Paton, an African plant, is mainly used to treat microbial infections in traditional Burundian medicine. From the ethyl acetate extract of the aerial parts, five pentacyclic triterpenoid acids were isolated and characterized. Based on spectral analysis, these compounds were elucidated to be 2α, 3α, 19β-trihydroxyurs-12-en-28-oic acid (that was named jeremic acid) (**1**), 3β-hydroxyurs-12-en-28-oic acid (ursolic acid) (**2**), 2α, 3β-dihydroxyurs-12-en-28-oic acid (corosolic acid) (**3**), 2α, 3β, 19α-trihydroxyurs-2-en-28-oic acid (tormentic acid) (**4**) and 19-hydroxy-2-hydroxymethyl norursa-2, 12-dien-28-oic acid (hyptadienic acid) (**5**). Ursolic (MIC = 17.5–68 μM) and corosolic (MIC = 17–68 μM) acids showed significant antibacterial activities against both the Gram-positive *Staphylococcus aureus* (methicillin-susceptible and -resistant strains) and the Gram-negative *Escherichia coli*, which may substantiate the use of *P. rotundifolium* in traditional Burundian medicine. Such hydroxylated pentacyclic triterpenoid acids could point to new antimicrobial strategies that may help overcoming the antimicrobial resistances actually observed throughout the world.

1. Introduction

Infectious diseases remain to this day one of the major public health problems that concerns the whole world. Over 10 years ago, the World Health Organization (WHO) estimated these diseases were the leading cause of morbidity and mortality worldwide, accounting for about half of deaths in tropical countries (WHO, 2003). Even if some improvements are noticeable, the situation remains very alarming. Indeed, according to the latest report by the WHO on global health statistics (WHO, 2014), infectious diseases cause life expectancy to decrease by 70% in the WHO African Region and by 8% in high-income countries. Moreover, apart from the fact that significant progress has been made against child deaths after the first month of birth (measles, −80%; HIV/AIDS, −51 %; diarrhea, −50%; pneumonia, −40%, and malaria, −37%), half of the top 20 causes of premature death worldwide are associated with infectious diseases, complicated by other factors, maternal, neonatal or nutritional (WHO, 2014).

The resistance of microorganisms to existing conventional antimicrobial drugs represents an equally important issue impeding treatments. The antibiotic era (1940–1970) commonly known as "the golden age" (the period during which most of today's antibiotics were discovered) was promising but, unfortunately, resistances have rapidly occurred, favored by major selective

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pressures mainly due to clinical abuse in humans and overuse in animal feed and aquaculture (Looke et al., 2013; O'Neill, 2014; Wang and Schaffner, 2011). A return to the pre-antibiotic period appears as a dreadful possibility, with a major risk of untreatable diseases increase; O'Neill (2014) estimates that over 10 million people would die every year within the next 35 years because of these antimicrobial resistance problems.

Medicinal plants have long been used against a number of diseases (Petrovska, 2012), notably infections (Cowan, 1999; Iwu et al., 1999; Ngezahayo et al., 2015; Rios and Recio, 2005). Indeed, part of their secondary metabolites present antimicrobial activities, either direct (bactericide or bacteriostatic) (Kim and Ausubel, 2006) or indirect (reversion of resistances, modulation of quorum sensing) (Okusa et al., 2007; Rasamiravaka et al., 2015b). These could certainly help to address the current problems of antimicrobial resistance (Hatano et al., 2005; Okusa et al., 2009; Rasamiravaka et al., 2015a).

In fact, the interest for herbal medicines remains important: (i) in its strategy for 2014–2023, the WHO encourages the development and modernization of traditional medicine as an integral part of emerging healthcare systems (WHO, 2013); (ii) Newman and Cragg (2012), confirming previous studies (Rout et al., 2009; Wachtel-Galor and Benzie, 2011), reported that about 50% of the modern medicines developed over 1981–2010 were natural substances or were inspired by natural substances.

In spite of its negation during the decades of colonization, Traditional African Medicine (TAM) has resisted and remains extremely vivid in Africa (Kahumba et al., 2015), notably in Burundi. Indeed, TAM represents the primary health care needs for 80% of both rural and urban population, be it in daily or emergency cares (Kasilo et al., 2010). The main motivations for its use are the cultural attachment, the confidence in its efficacy but also the scarcity and cost of quality modern drugs (Elujoba et al., 2005). TAM is mainly based on plants, but also on some animal and mineral products.

In an ethnobotanical survey conducted recently on medicinal plants used against infections by traditional healers and herbalists of the city of Bujumbura, capital of Burundi (Ngezahayo et al., 2015), *Platostoma rotundifolium* (Briq.) A. J. Paton (perennial herb with a small bush, of the Lamiaceae family and up to 1.5 m high) was the most used species, cited by 75% of interviewees. It is primarily used against microbial infections, including skin disorders. In addition, a preliminary phytochemical study carried on the aerial parts of this species showed antibacterial activity against Gram-positive and −negative bacteria, antibiotic-sensitive and resistant; the most active fractions were the aerial parts dichloromethane and ethyl acetate extracts (Ngezahayo et al., 2014).

In the present work, the *P. rotundifolium* aerial parts ethyl acetate extract was subjected to bio-guided fractionation, applying TLC-bioautography (Okusa et al., 2010), to isolate and characterize different active molecules that may justify its use in traditional Burundian medicine and possibly provide a clue to new antimicrobial strategies.

2. Experimental

2.1. General experimental procedures

Column and flash chromatography were performed with silica gel 60 (40–63 μm, Merck, Germany) and pre-packed silica Redi*Sep*® R_f Colomn (Teledyne Isco, 4 g Flash Column, CV 4.8 ml, 18 ml/min, max. pressure 1200 psi (13.8 bar), 20–400 mg sample, USA), respectively. Thin Layer Chromatography (TLC) was carried out on precoated silica gel 60 F_{254} (Merck) plates eluted with dichloromethane-ethyl acetate (80:20), dichloromethane-methanol (96-4) or chloroform-methanol (93:7). The TLC plates were sprayed with 1% ethanolic vanillin, followed by 10% ethanolic surfuric acid and heated at 110 °C for 10 min. Preparative TLC (TLC silicagel 60 F_{254}, 20 × 20, Merck, Germany) were used during purification processes. TLC-bioautography was performed on 10 × 5 cm precoated silica gel 60 F_{254} glasses plates (Merck, Darmstadt, Germany). Melting points were measured with a Stuart equipment. IR spectra were recorded with an IRAffinity-1 FTIR spectrophotometer (Shimadzu). Optical rotation values were measured on a Perkin-Elmer 241 polarimeter. High-resolution ESI–MS were determined using an Agilent 6520 Accurate-Mass Q-TOF LC–MS (Palo Alto, CA, USA). NMR experiments (^{1}H, ^{13}C (BBD and Dept 135), COSY, HSQC, HMBC and NOESY) were performed on Bruker Avance 300, 400 or 600 MHz.

2.2. Plant material

Fresh aerial parts of *P. rotundifolium* (Briq.) A. J. Paton were collected in July 2012 from Nyabiraba area (1730 m, S 03.45325°, E 029.47607°) in Bujumbura Rural Province (Burundi). The plant was identified by the specialists of the Herbarium of the National Botanical Garden of Meise (Belgium) where a voucher specimen has been deposited under the number BR0000013315900.

2.3. Extraction and isolation

Powdered aerial parts (1.7 kg) were percolated successively with 8 l of each of five solvents: *n*-hexane (yield, 16.4 g), dichloromethane (yield, 49.9 g), ethyl acetate (yield, 18.4 g), methanol (yield, 52.8 g) and water (yield, 125.6 g). The most active fraction, the ethyl acetate extract (18.4 g), was subjected to fractionations by column chromatography (360 g silica; 80 × 6 cm i.d), eluting with 10.5 l of dichloromethane-ethyl acetate mixtures with increasing polarities (10 to 100% EtOAc). The fractions were pooled according to their TLC profile to give eight fractions (FI-FVIII) with respective yields 0.92, 0.11, 0.10, 1.47, 1.20, 0.95, 3.34 and 4.47 g.

Portions of 3 active fractions (420 mg of FIV, 890 mg of FV and 849 mg of FVIII) were subjected to flash chromatography, eluting with dichloromethane-ethyl acetate gradients (0 to 100% EtOAc). The obtained active subfractions were purified by preparative TLC using chloroform-methanol (93:7) and dichloromethane-methanol (96:4) as mobile phases, yielding compounds (**1**) (23 mg), (**2**) (42 mg), (**3**) (10 mg), (**4**) (30 mg) and (**5**) (8 mg).

Compound **1** (Jeremic acid): white powder; R_f (eluent: dichloromethane/methanol, 96:4) = 0.12; mp 245 °C; $[\alpha]_D^{20}$ + 20.5° (*c* = 0.07, methanol); IR (cm^{-1}) = 3566, 2936, 2874, 2682, 1455, 1288, 1222, 1160, 1038, 938; ^{1}H- and ^{13}C— NMR data, see Tables 1 and 2; ESI-HRMS (positive mode): *m/z* 489.3576 $[M+H]^+$ (theoretical *m/z*, 489.3575); 506.3836 $[M+NH_4]^+$ (theoretical *m/z*, 506.3840); 511.3395 $[M+Na]^+$ (theoretical *m/z*, 511.3394); 527.3174 $[M+K]^+$ (theoretical *m/z*, 527.3133) (See supplementary data); ESI-HRMS/MS (positive mode): *m/z* (rel. int.) 489.3552 $[M+H]^+$ (75), 471.3454 $[(M+H)-H_2O]^+$ (96), 453.3348 $[(M+H)-2H_2O]^+$ (65), 425.3405 (56), 407.3285 $[C_{29}H_{43}O]^+$ (57), 219.1714 $[C_{15}H_{23}O]^+$ (18), 207.172 [retro-Diels-Alder (RDA) ion, $[C_{14}H_{23}O]^+$] (17), 205.1575 $[C_{14}H_{21}O]^+$ (83), 203.1788 $[C_{15}H_{23}]^+$) (7), 201.163$[C_{15}H_{21}]^+$ (100), 189.1636 (RDA ion − H_2O, $[C_{14}H_{21}]^+$) (17), 187.1473 $[C_{14}H_{19}]^+$ (41), 177.1627 $[C_{13}H_{21}]^+$ (14), 159.1159 $[C_{12}H_{15}]^+$ (13), 147.1159 $[C_{11}H_{15}]^+$ (28), 145.0986 $[C_{11}H_{13}]^+$ (10), 133.0996 $[C_{10}H_{13}]^+$ (13), 131.0857 $[C_{10}H_{11}]^+$ (13), 121.101 $[C_9H_{13}]^+$ (13), 119.0862 $[C_9H_{11}]^+$ (21), 107.086 $[C_8H_{11}]^+$ (11).

2.4. Bacterial strains

Five bacterial strains (four Gram-positive and one Gram-negative) were used in this work: *Staphylococcus aureus* (C 98506, C 100459, ATCC 33591 and ATCC 6538) and *Escherichia coli* ATCC

Table 1
600 MHz ^{1}H NMR Spectral data for compounds **1–5** [a] (δ ppm; *J* Hz). [a] **1–5** in pyridine-d_5; bs = broad singlet; d = doublet; dbs = doublet of broad singlet; dd = doublet of doublets; m = multiplet; s = singlet; t = triplet; td = triplet of doublets.

Position	1	2	3	4	5
1	1.91 (1β, dd, 1H, $^2J_{1\alpha-1\beta}$ = 12, $^3J_{1\beta-2}$ = 4); 1.77 (1α, t, 1H, $^2J_{1\alpha-1\beta}$ = $^3J_{1\alpha-2}$ = 12)	1.57 (m, 1H); 0.98 (m, 1H)	2.26 (1β, dd, 1H, $^2J_1$ = 12.3, $^3J_{1\beta-2}$ = 4.4); 1.30 (m, 1H)	2.27 (1β, dd, 1H, $^2J_{1\alpha-1\beta}$ = 12.4, $^3J_{1\beta-2}$ = 4.2); 1.33 (m, 1H)	4.58 (1a, d, 1H, $^2J_{1a-1b}$ = 14.9; 4.46 (1b, d, 1H, $^2J_{1a-1b}$ = 14.9)
2	4.32 (dbs, 1H, $^3J_{1\beta-2}$ = 12)	1.85 (m, 1H)	4.12 (m, 1H, $^3J_{1\alpha-2}$ = 11.2, $^3J_{1\beta-2}$ = 4.3)	4.12 (m, 1H)	/
3	3.77 (bs, 1H)	3.49 (dd, 1H, $^3J_{2\beta-3}$ = 10.5, $^3J_{2\alpha-3}$ = 5.5)	3.42 (d, 1H, $^3J_{2-3}$ = 9.3)	3.40 (d, 1H, $^3J_{2-3}$ = 9.3)	5.75 (bs, 1H)
4	/	/	/	/	/
5	1.69 (m, 1H)	0.89 (d, 1H, $^3J_{5-6\beta}$=11.5)	1.04 (m, 1H)	1.07 (m, 1H)	1.62 (m, 1H)
6	1.69 (m, 1H); 1.42 (m, 1H)	1.59 (m, 1H); 1.38 (m, 1H)	1.59 (m, 1H); 1.40 (m, 1H)	1,59 (m, 1H); 1.43 (m, 1H)	1.47 (m, 1H); 1.47 (m, 1H)
7	1.53 (m, 1H); 1.38 (m, 1H)	1.59 (m, 1H); 1.39 (m, 1H)	1.59 (m, 1H); 1.38 (m, 1H)	1.66 (m, 1H); 1.44 (m, 1H)	1.65 (m, 1H); 1.44 (m, 1H)
8	/	/	/	/	/
9	2.09 (m, 1H)	1,66 (m, 1H)	1.77 (m, 1H)	1.98 (m, 1H)	2.54 (dd, 1H, $^3J_{9\text{-}11trans}$ = 11.3, $^3J_{9\text{-}11cis}$ = 6.3)
10	/	/	/	/	/
11	2.17 (m, 1H); 2.10 (m, 1H)	1.97 (m, 2H)	2.03 (m, 1H); 2.00 (m, 1H)	2.16 (m, 1H); 2.1 (m, 1H)	2.46 (m, 1H); 2.39 (m, 1H)
12	5.6 (bs, 1H)	5.52 (bs, 1H)	5.48 (bs, 1H)	5.61 (bs, 1H)	5.62 (bs, 1H)
13	/	/	/	/	/
14	/	/	/	/	/
15	3.12 (td, 1H, $^3J_{15\text{-}16trans}$ = 13, $^3J_{15\text{-}16cis}$ = 4.3); 2.05 (m, 1H)	2.36; 1.23	2.35 (td, 1H, $^3J_{15\text{-}16trans}$ = 13.4, $^3J_{15\text{-}16cis}$ = 4.9); 1.21 (m, 1H)	2.36 (td, 1H, $^3J_{15\text{-}16trans}$ = 13.4, $^3J_{15\text{-}16cis}$ = 4.6); 1.32 (m, 1H)	2.37 (td, 1H, $^3J_{15\text{-}16trans}$ = 14.6, $^3J_{15\text{-}16cis}$ = 3.5); 1.30 (m, 1H)
16	2.35 (td, 1H, $^3J_{15\text{-}16trans}$ = 13.7, $^3J_{15\text{-}16cis}$ = 4.5); 1.28 (m, 1H)	2.15 (td, 1H, $^3J_{15\text{-}16trans}$ = 13.4, $^3J_{15\text{-}16cis}$ = 4.2); 2.03 (m, 1H)	2.13 (td, 1H, $^3J_{15\text{-}16trans}$ = 13.1, $^3J_{15\text{-}16cis}$ = 4.1); 2.02 (m, 1H)	3.15 (td, 1H, $^3J_{15\text{-}16trans}$ = 13.1, $^3J_{15\text{-}16cis}$ = 4.5); 2.07 (m, 1H)	3.14 (td, 1H, $^3J_{15\text{-}16trans}$ = 13.1, $^3J_{15\text{-}16cis}$ = 4.5); 2.06 (m, 1H)
17	/	/	/	/	/
18	3.05 (s, 1H)	2.67 (d, 1H, $^3J_{18-19}$ = 11.4)	2.65 (d, 1H, $^3J_{18-19}$ = 11.5)	3.07 (s, 1H)	3.06 (s, 1H)
19	/	1.5 (m, 1H)	1.47 (m, 1H)	/	/
20	1.50 (m, 1H)	1.05 (m, 1H)	1.03 (m, 1H)	1.52 (m, 1H)	1.52 (m, 1H)
21	2.08 (m, 1H); 1.34 (m, 1H)	1.48 (m, 1H); 1.41 (m, 1H)	1.47 (m, 1H); 1.39 (m, 1H)	2.11 (m, 1H; 1.36 (m, 1H)	2.09 (m, 1H); 1.35 (m, 1H)
22	2.16 (m, 1H); 2.08 (m, 1H)	2.00 (m, 1H); 1.97 (m, 1H)	1.99 (m, 1H); 1.96 (m, 1H)	2.17 (m, 1H); 2.07 (m, 1H)	2.16 (m, 1H); 2.08 (m, 1H)
23	1.27 (s, 3H)	1.27 (s, 3H)	1.29 (s, 3H)	1.28 (s, 3H)	1.09 (s, 3H)
24	0.91(s, 3H)	1.05 (s, 3H)	1.09 (s, 3H)	1.10 (s, 3H)	0.99 (s, 3H)
25	0.99 (s, 3H)	0.91 (s, 3H)	1.00 (s, 3H)	1.03 (s, 3H)	1.18 (s, 3H)
26	1.12 (s, 3H)	1.08 (s, 3H)	1.07 (s, 3H)	1.13 (s, 3H)	1.17 (s, 3H)
27	1.65 (s, 3H)	1.25 (s, 3H)	1.23 (s, 3H)	1.73 (s, 3H)	1.71(s, 3H)
28	/	/	/	/	/
29	1.43 (s, 3H)	1.03 (d, 3H, $^3J_{19-29}$ = 6.4)	1.00 (d, 3H, $^3J_{19-29}$ = 6)	1.45 (s, 3H)	1.42 (s, 3H)
30	1.12 (d, 3H, $^3J_{20-30}$ = 6)	0.98 (d, 3H, $^3J_{20-30}$ = 6.3)	0.97 (d, 3H, $^3J_{20-30}$ = 6.4)	1.14 (d, 3H, $^3J_{20-30}$ = 7)	1.12 (d, 3H, $^3J_{20-30}$ = 6.7)

25922. The first three *Staphylococcus* strains were methicillino-resistant *S. aureus* (MRSA) whereas the last one was susceptible *S. aureus* (MSSA). The strains C98506 and C100459 were clinical isolates from the "Centre Hospitalier Universitaire de Charleroi" (Belgium) and the ATCC strains were obtained from the American Type Culture Collection.

2.5. Evaluation of minimum inhibitory (MIC) and bactericidal concentrations (MBC)

MIC and MBC were determined by broth microdilution method (Jorgensen and Turnidge, 2003). Briefly, each extract (or product) was dissolved in DMSO, diluted with Mueller-Hinton broth (MHB) to achieve a concentration less than 5% DMSO (v/v), transferred in 96-well plates (200 μl/well) and serially diluted (base 2 logarithmic dilutions) with MHB. Recent cultures (18–24 h) of bacterial strains were stirred with a physiological solution (0.85% NaCl), diluted to McFarland 0.5 turbidity (about 10^6 cells/ml) and inoculated in the 96-well plates. These cultures were then incubated at 37 °C for 24 h. After incubation, a solution of 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium (MTT) (0.8 mg/ml) was distributed into the wells (30 μl/well) for easier visualization of bacterial growth, and plates were further incubated for a 30 min. Thus, the MIC was detected with the naked eye as the lowest concentration of product that completely inhibits bacterial growth.

The MBC (the lowest bactericidal concentration of the extract/product, after 18–24 h incubation at 37 °C) was determined (before the MTT treatment) by transferring the sample from the wells showing no microbial growth into a fresh medium. The lowest concentration at which no microorganisms grow in the sub-culture was identified as the MBC. Negative and positive control tests were

Table 2
150 MHz ^{13}C NMR Spectral data for compounds **1–5** [ab] (ppm). [a] **1** in pyridine-d_5 and in DMSO-d_6; [b] **2–5** in pyridine-d_5; [c] hidden by DMSO.

Position	1		2	3	4	5
	Pyridine-d_5	DMSO-d_6				
1	43.3	41.2	39.5	48.5	48.3	61.3
2	66.5	64.7	28.6	69.0	69.0	157.2
3	79.8	77.9	78.5	84.2	84.3	134.0
4	39.2	38.0	39.9	40.3	40.3	42.4
5	49.2	47.7	56.2	56.3	56.4	64.1
6	33.9	32.6	19.2	19.3	19.4	18.1
7	19.0	17.7	34.0	33.9	33.9	35.0
8	41.0	[c]	40.4	40.4	40.8	42.9
9	48.0	46.5	48.5	48.5	48.2	44.1
10	39.1	37.8	37.9	38.9	38.9	51.3
11	24.5	23.2	24.1	24.1	24.5	27.5
12	128.4	126.8	126.1	125.9	128.3	128.6
13	140.4	138.6	139.7	139.7	140.4	140.7
14	42.6	41.4	42.9	42.9	42.6	42.8
15	26.8	25.2	29.1	29.0	29.8	30.1
16	29.7	28.0	25.3	25.3	26.8	26.8
17	48.7	46.9	48.5	48.4	48.7	48.7
18	55.0	53.2	53.9	53.9	55.0	55,21
19	73.1	71.6	39.8	39.9	73.1	73.0
20	42.8	41.6	39.8	39.8	42.8	42. 8
21	27.3	25.2	31.5	31.5	27.4	27.4
22	38.9	37.3	37.7	37.9	38.9	38.9
23	29.9	28.9	29.2	29.8	29.7	30.5
24	22.7	21.8	17.0	17.4	18.1	22.2
25	17.0	16.3	16.1	17.9	17.3	19.4
26	17.7	16.6	17.9	17.9	17.7	19.3
27	25.0	24.1	24.3	24.3	25.1	25.8
28	181.1	178.9	180.3	180.3	181.1	181.0
29	27.5	26.4	17.9	18.1	27.5	27.5
30	17.2	16.1	21.8	21.8	17.3	17.2

similarly performed with medium alone (MHB), DMSO and antibiotic solutions.

2.6. Bioautography assay

TLC-bioautography was realized following the optimized method described by Okusa et al. (2010) and instructions reviewed by Choma and Grzelak (2011) and Marston (2011). Briefly, 40 μg of extract (4 mg/ml, 10 μl) or 10 μg of each solubilized fraction (1 mg/ml, 10 μl) were deposited on a silica plate. Then, the plate was placed in a developing tank, presaturated with dichloromethane-ethyl acetate (80:20) as eluent solvent system. After elution and evaporation of solvent (by leaving the TLC plate in a hood overnight at room temperature), a mixture of bacterial suspension (0.5 McFarland) and Mueller-Hinton medium (in the proportions 1: 9) was sprayed on the developed TLC plate, that was then incubated for 24 h at 37 °C.

Finally, MTT solution (0.8 mg/ml) was sprayed over the plate and a second incubation was carried out for 4 h at 37 °C. Active compounds were observed as clear spots (characteristic of bacterial growth inhibition) against a purple background (Figs. 1 and 2).

In parallel to MIC and MBC evaluations, direct-bioautography tests (Fig. 3) were carried out by spotting, on a silica plate, amounts of solubilized extracts ranging from 1 to 20 μg. This TLC plate was then treated as described, except for the migration step.

3. Results and discussion

3.1. Antibacterial activity of the ethyl acetate extract and its fractions

A previous work (Ngezahayo et al., 2014) indicated that the ethyl acetate extract of *P. rotundifolium* aerial parts has significant antibacterial activities (62.5–250 μg/ml) compared to

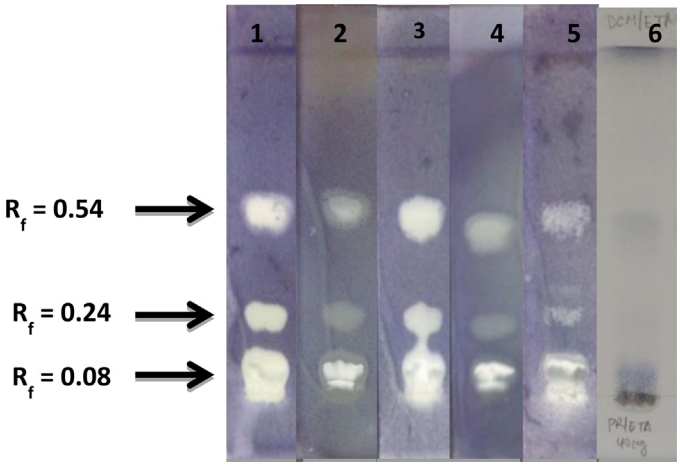


Fig. 1. TLC-bioautography of the ethyl acetate extract of *Platostoma rotundifolium* aerial parts (40 μg). Mobile phase: dichloromethane - ethyl acetate (80:20). Bacterial strains: (1) MRSA C100459; (2) MSSA ATCC6538; (3) MRSA ATCC 33591; (4) MRSA C98506; (5) *E. coli* ATCC 25922. Spots visualization: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) (1–5); Vanillin-sulfuric acid reagent and observed in visible light (6); White spots = inhibition zones of bacterial growth.

other extracts of this species. TLC-bioautography of this extract revealed identical antibacterial compounds (similar R_f values) active both on the Gram-negative *E. coli* and the Gram-positive *Staphylococcus* species (Fig. 1). Fractionation of this extract on a silica column yielded eight fractions (FI-FVIII) (Table 3, Figs. 2 and 4). Antibacterial tests (MIC, MBC and TLC-bioautography) showed that the first three fractions (FI-FIII) were inactive (MIC/MBC ≥ 500 μg/ml and absence of inhibition zones on TLC plate) while the following five fractions (FIV-FVIII) were active (Table 3, Fig. 2). However, although the FV and FVIII fractions have shown antibacterial activity by TLC-bioautography, their MIC and MBC remain above 500 μg/ml; this would be due to problems in solubilizing these fractions in DMSO and in MHB. Some column fractions (FIV, FVI and FVII) were active with MIC and MBC ranging from 16 to 62 μg/ml; similar MIC and MBC suggest that these fractions contain products with bactericidal effects on all tested bacterial strains. TLC-bioautography revealed that FV contains more antibacterial compounds (at least three white spots) compared to the other active fractions and FVI-FVII probably contain the same antibacterial compound (identical R_f values) (Fig. 2). Thus, FIV, FV and FVII were selected for further fractionation and isolation of antibacterial compounds.

3.2. Isolation and identification of active compounds

Fractions FIV, FV and FVII were submitted to flash chromatography on pre-packed silica Redi*Sep*® R_f Columns and purified with preparative TLC to afford a new (compound **1**) and four known pentacyclic triterpenoid acids (compounds **2**–**5**). The structure of the known compounds were determined using NMR (^1H, ^{13}C (BBD and Dept 135), COSY, HSQC and HMBC) and high-resolution (HR) MS experiments as well as by comparison with literature data. The compounds were identified as ursolic acid (**2**) (Seebacher et al., 2003), corosolic acid (**3**) (Hou et al., 2009; Zong and Zhang, 2013), tormentic acid (**4**) (Numata et al., 1989) and hyptadienic acid (**5**) (Taniguchi et al., 2002) (Fig. 5).

Compound **1** was isolated as a white powder (23 mg) soluble in pyridine. Its HRESI–MS showed a quasi-molecular ion peak $[M+H]^+$ at *m/z* 489.3576 (calculated for $C_{30}H_{49}O_5$, 489.3575) (Fig. 6), suggesting that the compound molecular formula is $C_{30}H_{48}O_5$. The infrared spectrum showed absorptions bands at 3400 (hydroxyl groups), 2936 (methine group) and 1682 cm^{-1} (carbonyl group),

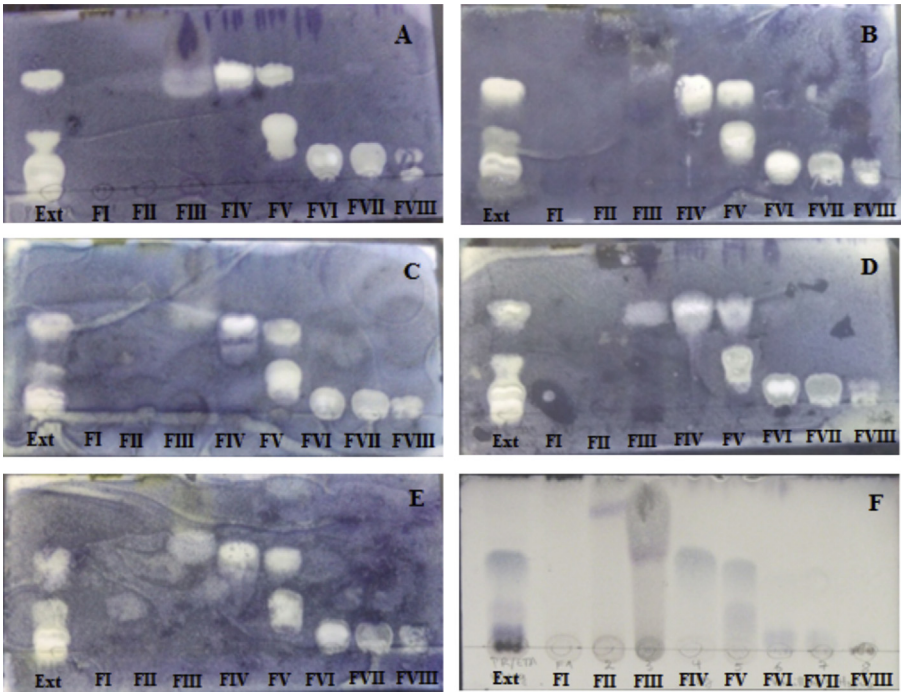


Fig. 2. TLC-bioautography of fractions obtained from the ethyl acetate extract of *Platostoma rotundifolium* aerial parts. Amount deposited on the plate: 40 μg (extract) and 10 μg (fractions). Mobile phase: dichloromethane - ethyl acetate (80:20). Strains: MRSA C100459 (A), MRSA C98506 (B), MRSA ATCC 33591 (C), MSSA ATCC 6538 (D) and *E. coli* ATCC 25922 (E). Spots visualization: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) (A-E); Vanillin-sulfuric acid reagent and observed in visible light (F); FI-FVIII = Fractions from the column chromatography; White spots = inhibition zones of bacterial growth.

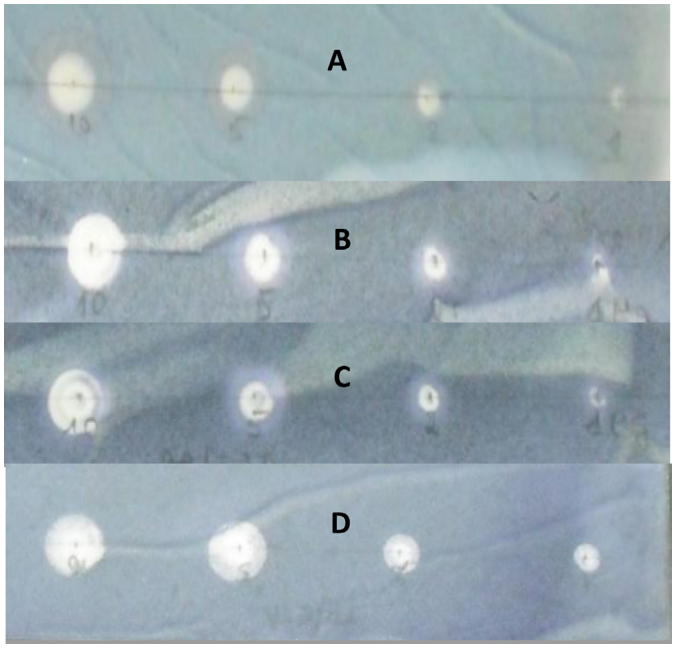


Fig. 3. Direct-bioautography of the ethyl acetate extract of *Platostoma rotundifolium* aerial parts. (A) MRSA C98506; (B) MRSA C100459; (C) MSSA ATCC6538; (D) *E. coli* ATCC 25922; extract amount deposited on the plate (left to right): 10; 5; 2 and 1 μg.

which was confirmed by NMR data. As the number of degrees of unsaturation is seven, it was concluded that the molecule **1** could contain five rings, a carbonyl group and a double bond. This was supported by the ^{13}C NMR (BBD and DEPT) spectra which indicated the presence of seven methyl groups, eight methylenes, seven methines [including two olefinic carbons at δ_C 128.4 (C-12) and 140.4 (C-13)] and eight quaternary [carbonyl at δ_C 181.1 (C-28) and an oxygenated δ_C 73.1 (C-19)] carbons (Table 2). The ^1H NMR spectrum of **1** also showed signals for seven methyls comprising six singlets [δ_H 1.65 (3H, s), 1.43 (s, 3H), 1.27 (3H, s), 1.12 (3H, s), 0.99 (3H, s) and 0.91 (3H, s)] and one doublet [δ_H 1.12 (3H, d, $^3J_{20-30} = 6$)], a vinylic proton [δ_H 5.6 (1H, bs)] and two oxymethines [δ_H 4.32 (dbs, 1H, $^3J_{1\beta-2} = 12$) and 3.77 (bs, 1H)] (Table 1). All of the above together suggested that the compound **1** is an ursane triterpenoid type. Detailed analysis of 1D and 2D NMR spectra (^1H, ^{13}C, COSY, HSQC and HMBC) of **1** showed a strong resemblance to

Table 3
Antibacterial activity of fractions obtained from the ethyl acetate extract of *Platostoma rotundifolium* aerial parts.

Fractions of ETA extract	Minimum inhibitory (bactericidal) concentrations (μg/ml)									
	MRSA C98506		MRSA C100459		MRSA ATCC 33591		MSSA ATCC6538		*E. coli* ATCC 25922	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
FI	>500	>500	>500	>500	>500	>500	500	500	>500	>500
FII	>500	>500	>500	>500	500	500	500	500	>500	>500
FIII	>500	>500	>500	>500	>500	>500	500	500	>500	>500
FIV	16	16	16	16	16	16	16	16	31	31
FV	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
FVI	62	62	62	62	31	31	62	62	62	62
FVII	31	31	62	62	31	31	31	31	62	62
FVIII	>500	>500	>500	>500	>500	>500	>500	>500	>500	>500
Cefotaxime[a]	64	64	64	64	>64	>64	16	16	16	16
Tetracycline[a]	1	–	1	–	>64	–	0.25	–	0.25	–

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MRSA: methicillino-resistant *Staphylococcus aureus*; MSSA: methicillino-sensitive *Staphylococcus aureus*.

[a] Positive control; MICs and MBCs values were evaluated from at least three experiments in triplicate.

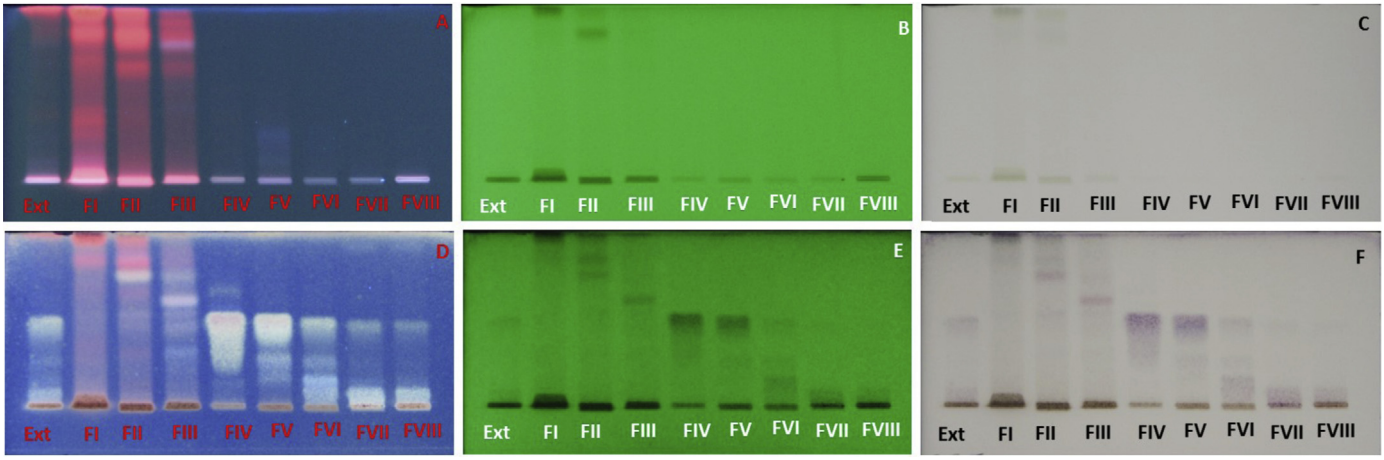
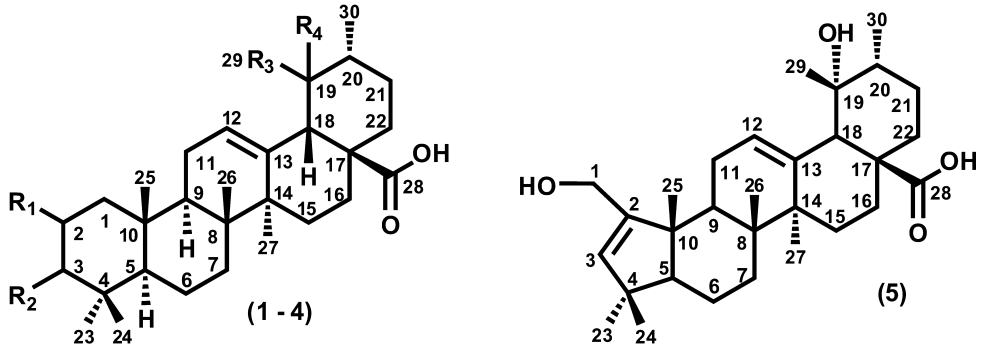


Fig. 4. TLC of fractions obtained from the ethyl acetate extract of *Platostoma rotundifolium* aerial parts. Amount deposited on the plate: 10 μg (10 μl, 1 mg/ml). Mobile phase: chloroform - ethyl acetate (80:20). Spots visualization under UV_{365nm} (A), UV_{254nm} (B) and visible light (C); derivatization with a vanillin-sulfuric acid reagent (heating 105 °C, 5–10 min) and visualization under UV_{365nm} (D), UV_{254nm} (E) and visible light (F). FI-FVIII = Fractions from the column chromatography; Ext. = Ethyl acetate extract.



Compounds	R_1	R_2	R_3	R_4
1	αOH	αOH	αCH_3	βOH
2	H	βOH	βCH_3	H
3	αOH	βOH	βCH_3	H
4	αOH	βOH	βCH_3	αOH

Fig. 5. Structure of compounds **1–5**.

those of euscaphic acid (Rocha et al., 2007; Takahashi et al., 1974). This is particularly observed by HMBC correlations between H-1 [δ_H 1.91 (1α, dd, 1H, $^2J_{1\alpha\text{-}1\beta}$ = 12)] and C-2 (δ_C 66.5), C-1 (δ_C 43.3) and H-3 [δ_H 3.77 (bs, 1H)], H-1 and C-3 (δ_C 79.8) and between H-3

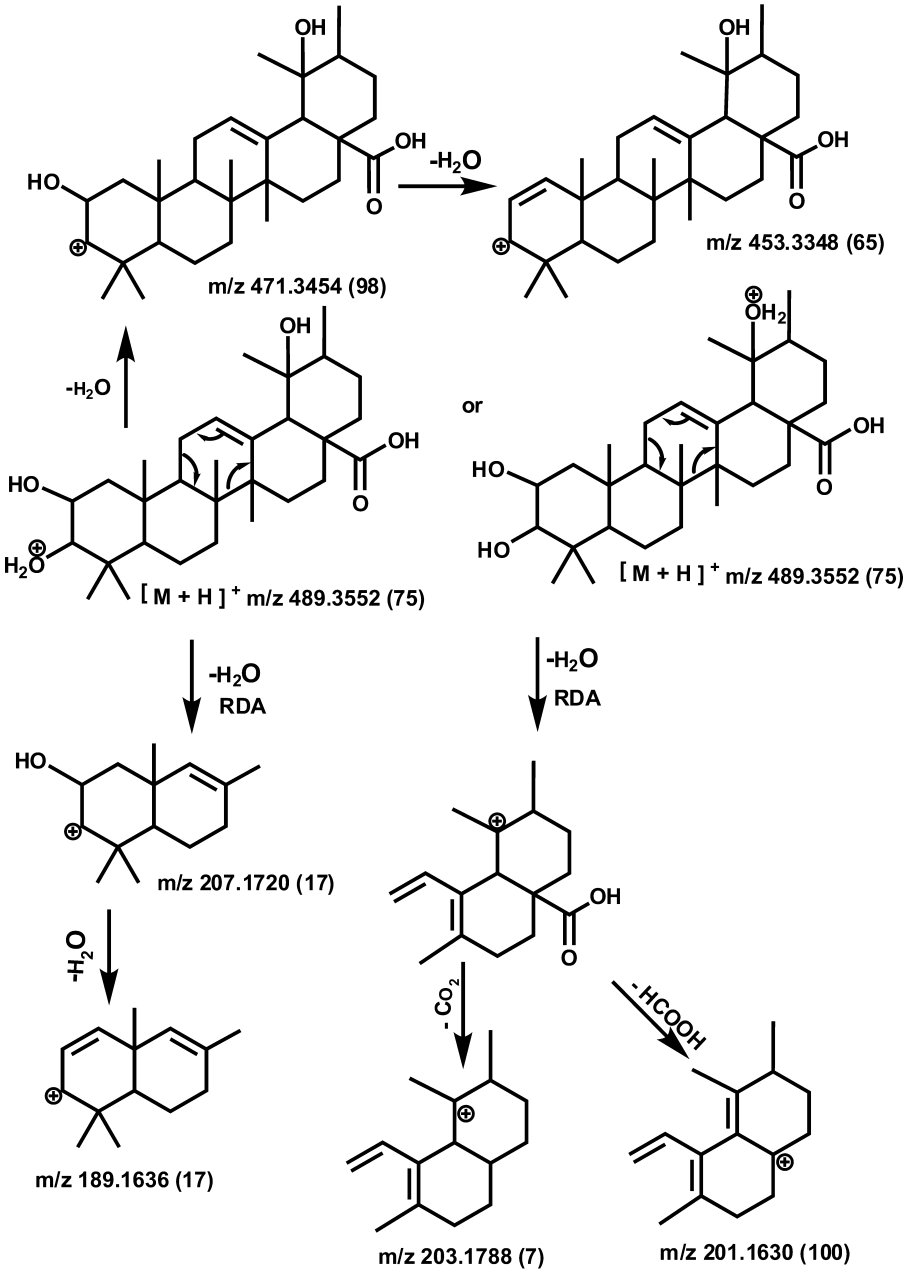


Fig. 6. Some possible mass spectral fragments of compound **1.**

and C-2 (Fig. 6). The obvious difference between euscaphic acid and compound **1** has been shown by other correlations observed in the NOESY spectrum (Fig. 7) especially between H-18 [δ_H 3.06 (s, 1H)] and H-20 [δ_H 1.52 (m, 1H)], H-20 and H-21 [δ_H 2.09 (m, 1H); 1.35 (m, 1H)], H-21 and H-30 [δ_H 1.12 (d, 3H, $^3J_{20-30}$ = 6.7)], and between H-30 and H29 [δ_H 1.43 (s, 3H)]. This indicated that the structure of **1** corresponds to 2α, 3α, 19β-trihydroxyurs-12-en-28-oic acid. As this compound was isolated and characterized for the first time, it was named "jeremic acid".

3.3. Antibacterial activity of isolated compounds

At least three active spots (R_f 0.08, 0.24 and 0.54) were visible on the TLC-bioautogram of the ethyl acetate extract (Fig. 1). The bioguided fractionation led to the isolation of five compounds (**1**–**5**), including the three active in bioautography test (Fig. 1). These three active compounds correspond to compounds (**2**) (R_f 0.54), (**3**) (R_f 0.24) and (**4**) (R_f 0.08) (Fig. 1). All the isolated compounds were also tested for their antibacterial activity by a microdilution method with oxacillin and streptomycin as positive controls. Compound **2** was active with the same values for MIC and MBC, which means that it has a bactericidal effect on all the five tested strains (MIC/MBC = 8–31 μg/ml or 17.5–68 μM, Table 4). These results are indeed consistent with the literature data on the antibacterial activity of compound **2** on sensitive (Kurek et al., 2012) and resistant bacteria (Horiuchi et al., 2007). Compound **3** was also bactericidal on all the tested strains (MIC/MBC = 8–32 μg/ml or 17–68 μM) (Table 4). Compounds **2** and **3** showed significant activity against resistant strains as their MIC/MBC values were at least 8 times smaller compared to oxacillin (Table 4). Comparing MIC and MBC values indicates that **3** could be a bit more active than **2**. This is consistent with literature data yielding a higher activity for corosolic acid compared to ursolic acid, especially in Gram-positive bacteria (Shai et al., 2008; Zhang et al., 2007). Compound **4** was less active

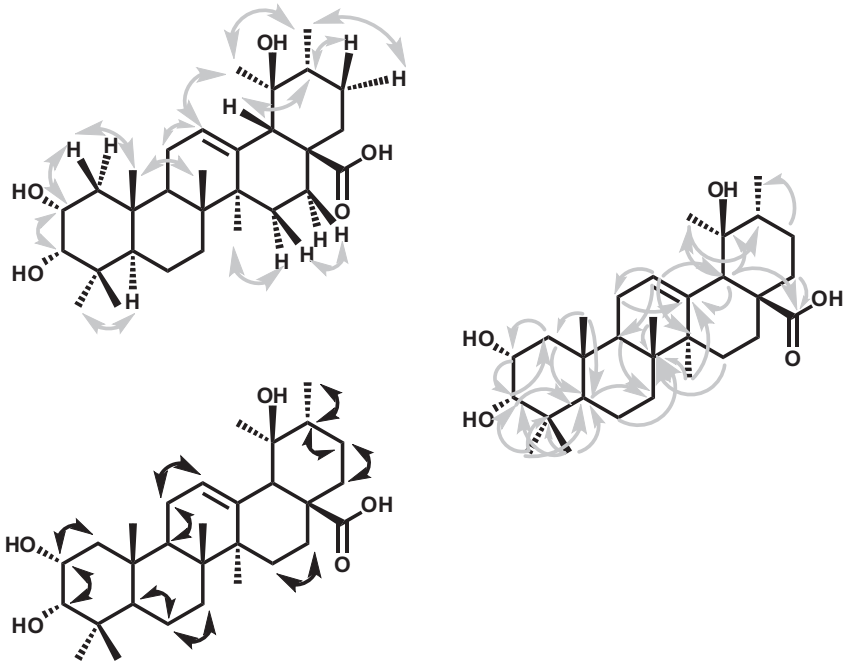


Fig. 7. Key COSY (H ⌒ H), NOESY (H ⌒ H) and HMBC (H ⌒ C) correlations of compound **1**.

Table 4
Antibacterial activity of isolated compounds from the ethyl acetate extract of *Platostoma rotundifolium* aerial parts.

Compounds	Minimum inhibitory (bactericidal) concentrations (μg/ml and *μM in italic*)									
	MRSA C98506		MRSA C100459		MRSA ATCC 33591		MSSA ATCC6538		*E. coli* ATCC 25922	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
	>524	*>524*	*>524*	*>524*	*>524*	*>524*	*>524*	*>524*	*>524*	*>524*
2	8	8	16	16	16	16	16	16	31	31
	17.5	*17.5*	*35*	*35*	*35*	*35*	*35*	*35*	*68*	*68*
3	16	16	16	16	8	8	16	16	32	32
	34	*34*	*34*	*34*	*17*	*17*	*34*	*34*	*68*	*68*
4	128	128	128	128	128	128	64	64	256	256
	262	*262*	*262*	*262*	*262*	*262*	*131*	*131*	*524*	*524*
5	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
	>545	*>545*	*>545*	*>545*	*>545*	*>545*	*>545*	*>545*	*>545*	*>545*
Oxacillin[a]	> 64	–	64	–	>64	–	0.5	–	4	–
	>158	–	*158*	–	*>158*	–	*1.2*	–	*10*	–
Streptomycin[a]	2	–	2	–	>64	–	1	–	4	–
	3.4	–	*3.4*	–	*>110*	–	*1.7*	–	*6.8*	–

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MRSA: methicillino-resistant *Staphylococcus aureus*; MSSA: methicillino-sensitive *Staphylococcus aureus*.

[a] Positive control, MICs and MBCs values were evaluated from at least three experiments in triplicate.

with MIC/MBC values between 64–256 μg/ml (131–524 μM), but with a bactericidal effect (Table 4). The antibacterial activities of compounds **3** (Acebey-Castellon et al., 2011; Zhang et al., 2007) and **4** (Fru et al., 2013) are already described in the literature but, to our knowledge, this is the first time that their anti-MRSA activity is reported. By contrast, compounds **1** and **5** showed practically no antibacterial activity on any of the five tested strains (MIC/MBC ≥256 μg/ml) (Table 4).

As compounds **1**–**4** present the same basic skeleton, tentative elements could be drawn regarding their structure-activity relationship; according to MIC and MBC, they can be classified as follows: **3** > **2** > **4** > > > **1**. This suggests that the presence and configuration of the hydroxyl groups at C-2, C-3 and C-19 are important for antimicrobial properties.

Indeed, the addition of a hydroxyl group at C-2 on compound **2** yields compound **3** with increased activity. By contrast, the addition of a hydroxyl group at C-19 on the skeleton of compound **3** decreases the activity (compound **4**) which completely disappears when this C-19 hydroxyl group is in bêta position with the C-3 hydroxyl group in alpha (compound **1**). This importance of ring A hydroxylation is in line with the data of Tankeu Nyaa et al. (2009) who showed that a C-1 hydroxylation confers to 1β-hydroxyeuscaphic acid (1β, 2α, 3α-trihydroxyurs-12-en-28-oic acid), isolated from the seeds of *Butyrospermum parkii* (G.Don) Kotschy (a synonym of *Vitellaria paradoxa* C.F.Gaertn), a higher antimicrobial effect compared to compounds with the same skeleton. This is also consistent with very recent results of Huang et al. (2015) who synthesized several pentacyclic triterpenic acids and speculated that the introduction of an hydroxyl groups on the first ring (ring A) would increase their antimicrobial activity; this appeared especially important when the acid function (C-28) is esterified by small alkyl groups having one to four carbon atoms.

It was not surprising to isolate ursolic acid (UA) from *P. rotundifolium* because previous studies had already identified its presence

in the genus Platostoma (Aladedunye et al., 2008). Furthermore, this acid (3β-hydroxyurs-12-by-28-oic acid) and its isomer oleanolic acid (OA, 3β-hydroxyolean-12-by-28-oic acid) have long been described (Janicsak et al., 2006; Liu, 1995) and were found in many plant species (Kamatou et al., 2007; Liu, 2005, 1995). Although OA has not been isolated from the plant, it has been reported that these two triterpenoids acids frequently occur together because they share similar structural characteristics (Jesus et al., 2015), and therefore could be found in the same plant species, especially in Lamiaceae family (Janicsak et al., 2006). Indeed, the literature indicates that the ursane (from which derives UA) occurs from oleane (from which derives OA) by simple migration of the methyl group (C-29) from C-20 to C-19 (Breitmaier, 2006). Their antimicrobial activities were also reported many times by several researchers. For example, these acids have displayed antibacterial (Fontanay et al., 2008; Jesus et al., 2015; Wolska et al., 2010), antiviral (Jesus et al., 2015; Kong et al., 2013) antifungal (Tang et al., 2014) and antiprotozoal (Jesus et al., 2015) properties.

OA and UA are active against many bacterial species such as mycobacteria (Jiménez-Arellanes et al., 2013) and resistant-bacteria including MRSA and VRE (Horiuchi et al., 2007). These compounds are also able to inhibit the biofilm formation in cariogenic microorganisms (Zhou et al., 2013) and to enhance the activitity of bêta-lactam antibiotics to combat bacterial infections caused by some Gram-positive pathogens (Kurek et al., 2012). These interesting activities of these acids could be responsible for the antimicrobial activities most often observed in the plant extracts containing these plants products (Bisioa et al., 2015; Kamatou et al., 2007; Ngezahayo et al., 2014; Omotayo and Borokini, 2012; Tan et al., 2002), and could explain the use of plants containing OA and UA in traditional medicines of several countries (Kamatou et al., 2007; Ngezahayo et al., 2015; Tan et al., 2002).

4. Concluding remarks

This study is the first report on the phytochemistry of *P. rotundifolium* aerial parts, presenting the isolation and structure elucidation of a new and four known pentacyclic triterpenic acids. Among these, corosolic and ursolic acids showed significant activity against all tested bacterial strains, both sensitive and resistant (MRSA); these findings support the use of the plant in traditional Burundian medicine.

MRSA being the cause of many infections, notably nosocomial, hydroxylated pentacyclic terpenoid derivatives (including corosolic and ursolic acids) give a clue in the search for new antimicrobial compounds that could overcome the antimicrobial resistances actually observed throughout the world.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2016.04.004.

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