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Antimicrobial activity of pentacyclic triterpenes isolated from African Combretaceae

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Abstract

Four pentacyclic tritepenes were isolated from *Combretum imberbe* Engl. & Diels, of which two are novel glycosidic derivatives of 1α,3β,23-trihydroxyolean-12-en-29-oic acid (hydroxyimberbic acid). *Terminalia stuhlmannii* Engl. & Diels stem bark yielded two glycosides of hydroxyimberbic acid, one of which is reported for the first time. The structures of the isolated compounds were elucidated by spectroscopic methods. Several of the compounds had antibacterial activity, imberbic acid showing particularly potent activity against *Mycobacterium fortuitum* and *Staphylococcus aureus*.

Keywords: Combretaceae; *Combretum imberbe*; *Terminalia stuhlmannii*; Pentacyclic triterpenoids; Antimicrobial activity

1. Introduction

The Combretaceae is a large family with at least 600 species (Hutchings et al., 1996; Exell, 1970). The two most commonly occurring genera are *Combretum* and *Terminalia,* each with 250 species; species of both are widely used in African traditional medicine. We report the isolation of a total of five compounds from *Combretum imberbe* Engl. & Diels and *Terminalia stuhlmannii* Engl. & Diels, of which three were isolated for the first time.

Combretum imberbe grows up to 30 m tall (usually 6–10 m) or as a shrub with spiny/spiky branchlets and silvery leaves, opposite or subopposite (Exell, 1970). It is found in sandy karoo soils of the African savanna. Rogers and Subramony (1988) have isolated pentacyclic triterpene acids from the leaves. Related glycosides have also been isolated, all based on the olean-12-en-29-oate aglycone which has been given the trivial name imberbic acid (Rogers, 1988). In this study, imberbic acid was isolated from *C. imberbe*, together with three glycosides of the 23-hydroxy derivative of imberbic acid, of which two are novel.

T. stuhlmannii is a small tree or shrub up to 12 m high with brownish-grey or whitish-grey bark and leaves clustered at the ends of spur shoots. It is found in *Acacia* savanna in hotter drier areas and had not been previously investigated. Related genera have yielded ursane-type triterpenes (Panzini et al., 1993; Kumar and Prabhakar, 1987; Tripathi et al., 1992). We report the isolation of two pentacyclic triterpenes from the stem bark of *T. stuhlmannii* which are based on the 23-hydroxylated prototype aglycone of *C. imberbe,* providing evidence, for the first time, of a chemotaxonomic link between *Combretum* and *Terminalia*. One of the compounds isolated from *T. stuhlmannii,* a bidesmoside, is reported for the first time.

2. Results and discussion

The leaves of *C. imberbe* were collected, sun-dried and ground then defatted with *n*-hexane to remove very non-polar components which could block the HPLC column. After extraction with dichloromethane (DCM), the relatively non-polar constituents were removed by use of Dialon HP-20 and then size exclusion chromatography with Sephadex LH-20. HPLC fractionation resulted in four triterpenoids.

For *T. stuhlmannii*, the stem bark was collected; there was little leaf material available at the time of collection.

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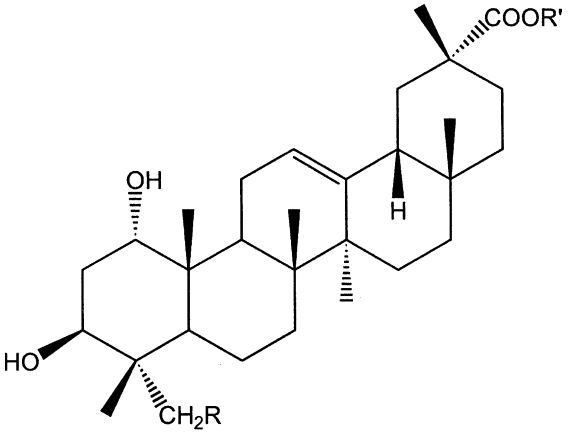
The procedure used for *C. imberbe* was also used on the sun-dried stem bark of *T. stuhlmannii*. Four compounds were isolated of which only two were sufficiently pure and are reported here.

A total of five 1,3-hydroxylated pentacyclic olean-12-ene triterpenoids (Fig. 1) were isolated from the two species by preparative HPLC. Compound **1** was isolated in a large quantity and from various fractions; this appears to be the major constituent in the leaves of *C. imberbe*. It had been previously isolated from the same species by Rogers and Subramony (1988). Hydroxyimberbic acid glycosides **2**, **3** and **4** were isolated from *C. imberbe;* **3** and **4** are reported here for the first time. Glycosides **2** and **5** were isolated from *T. stuhlmannii*.

Preliminary analysis of NMR data showed that the isoprenoids from both genera, *Combretum* and *Terminalia*, were triterpenoid-type compounds. From previous work, tetracyclic and pentacyclic triterpenes have been isolated from related species of Combretaceae (Rogers, 1988; Rogers and Subramony, 1988; Facundo et al., 1993; Panzini et al., 1993; Jossang et al., 1996; Rogers and Verotta, 1995). FTIR showed the presence, for all the compounds, of hydroxyl (ca. 3440 cm^{-1}), olefinic (ca. 1650 cm^{-1}), methyl (ca. 1460 cm^{-1}), geminal dimethyl (ca. 1380 cm^{-1}) (Biessels et al., 1974; Mahajan et al., 1995), and carboxylic acid (ca. 1680 cm^{-1}) or its esterified form (ca. 1730 cm^{-1}).

Compound **1** crystallized as a white-to-colourless solid from a Sephadex LH20 fraction after elution of a sample of *C. imberbe* with 100% trichloromethane (TCM). It was also isolated from other fractions by RP HPLC. The NMR spectra were suggestive of a triterpenoid-type compound with a carboxylic function, a trisubstituted double bond and an AB system. This pattern was seen in all the other compounds isolated from this plant suggesting a similarity in the chemical structure of the aglycone unit modified by various substituents.



Compound	R	R_1
1	H	H
2	4-Ac-O-Rh	H
3	3,4-Ac-O-Rh	H
4	3,4-Ac-O-Rh	Rh
5	4-Ac-O-Rh	Rh

Fig. 1. 1,3-Hydroxylated pentacyclic triterpenoids isolated in this study.

FABMS showed the pseudomolecular ion (M + 1) to be at *m/z* 473 (3%), suggesting the molecular formula $C_{30}H_{48}O_4$. Other prominent peaks were seen at m/z 453 (19%) $[M-H_2O]^+$, *m/z* 436 (6%) $[M-2H_2O]^+$, *m/z* 248 (10%) $[M-C_{14}H_{24}O_2]^+$, the base peak at *m/z* 188.9 (100%) $[M-C_{16}H_{27}O_4]^+$, *m/z* 130(45%) $[C_{10}H_{10}]^+$ and *m/z* 93 (74%) $[C_7H_9]^+$. These fragments were strongly suggestive of a hydroxylated Δ^{12}-pentacyclic triterpene. There is initial loss of water, then a Wagner–Meerwein rearrangement with subsequent loss of another water molecule (Geissman, 1959). The rearranged fragment then typically undergoes retro-Diels–Alder fragmentation (Djerassi et al., 1962; Karliner and Djerassi, 1966). This view was reinforced by the appearance in the ^{13}C NMR spectrum of six quaternary carbon atoms as well as signals at around δ_C l23 and l45 ppm which are characteristic of the Δ^{12}-oleanene skeleton (Doddrell et al., 1974; Amoros and Girre, 1987).

Using ^1H–^1H COSY, HMQC and HMBC we were able to build a detailed partial structure which was suggestive of a 1,3-dihydroxy-12-oleanen-29-oic acid. Comparison of the experimental data with the literature showed **1** to correspond to 1α,3β-dihydroxyolean-12-en-29-oic acid, previously isolated from the same species by Rogers and Subramony (1988) and given the trivial name imberbic acid. A very thorough NMR study confirmed the structure assigned previously: significant HMBC data are given in Table 1. Full ^{13}C and ^1H NMR assignments are given for all five compounds in Tables 2 and 3.

All the other fractions of both *C. imberbe* leaves and *T. stuhlmannii* stem bark were characterised as being glycosidic derivatives of 23-hydroxyimberbic acid. Their ^1H NMR and ^{13}C NMR spectra were virtually superimposable except in the region between δ_H 4.2 and 6 ppm (δ_C 65–79) where the resonances of the sugar moieties occur (Agrawal, 1992). ^1H–^1H COSY was used in all cases to unravel the bond connectivities which were then confirmed by ^2J and ^3J coupling correlations obtained from HMBC. In assigning the structures, the anomeric atoms were extremely useful, since they resonate in the uncrowded region spanning δ_H 4–6 in PMR and δ_C 90–112 ppm in CMR.

The sugar in every case was rhamnose which has its anomeric carbon normally around δ_C 101.5 ppm (Seidel, 1999) and is assumed to be the L-isomer on biogenetic grounds (Rogers, 1989). Modifications appeared largely around the point of attachment of the sugar.

Glycosylation at the carboxylate causes a downfield shift of the ^{13}C resonance (2–5 ppm) of the moiety, an upfield shift (0.5–2 ppm) of the adjacent resonance as well as a remarkable shielding of the anomeric carbon (δ_C 93–97 ppm; Agrawal, 1992). This along with HMBC correlations served to confirm the location of the sugar. The presence of more than one sugar was determined from the number of anomeric protons as these were not superimposed and also from the ^{13}C resonances of the oxymethines at δ_C 65–80 ppm.

Compound **2** was isolated from *C. imberbe* as a white amorphous powder. FABMS showed the presence of a molecular ion $[M+H]^+$ at *m/z* 677.5 which corresponds to $C_{38}H_{60}O_{10}$. Other prominent peaks appeared at *m/z* 658 (4 1%) $[M-H_2O]^+$, 472 (18%), 453 (80%), 437 (30), 248 (100), 231 (62). The other peaks were for the aglycone and show a similar pattern to that of **1**. The NMR spectra showed resonances in the sugar region but were otherwise similar to those of **1**, the most significant variations being around ring A: the resonances for C-3 and C-5 were most affected. In **1**, C-3 was deshielded by +4.2 ppm relative to that in **2**. More importantly C-5 was moved upfield by 6.7 ppm. The NMR data were suggestive of an acetylrhamnoside. The ^{13}C domain showed two carbonyl signals, one at δ_C 183.2, identical to that for the carboxylic acid in **1**, and another at δ_C 172.9 which correlated with the 4-H of the sugar in HMBC and thus confirmed the presence of the acetyl function. The position of the sugar was deduced from comparative analysis of NMR data, C-23 showing the glycosylation shift. Compound **2** was thus identified as 1α,3β-hydroxyimberbic acid 23-*O*-α-L-4-acetylrhamnopyranoside. This compound has been previously isolated from the leaves and fruit of *C. padoides* (Rogers, 1989; Panzini et al., 1993), but this is the first report of the isolation of this compound from the leaves of *C. imberbe* and the stem bark of *T. stuhlmannii*. It is also the first time that the presence of the aglycone is reported in the genus *Terminalia,* which has in the past yielded ursane based triterpenes or 28-carboxylated oleananes from the Asian species *T. arjuna* (Panzini et al., 1993; Kumar and Prabhakar, 1987).

Compound **3** was obtained as a yellow amorphous powder from *C. imberbe*. IR showed the presence of hydroxy (3450 cm^{-1}) and ester carbonyl (1732 cm^{-1}). **3** showed resonances similar to **1** and was very similar to **2**, but with two methyl signals at δ_H 2.05 attributable to two acetoxy groups. They were better resolved in the carbon domain at δ_C 21.2 and δ_C 21.3 with corresponding carbonyl resonances at ca. δ_C 172. FABMS showed a small pseudomolecular ion $[M+H]^+$ at *m/z* 719 which corresponds to the molecular formula $C_{40}H_{62}O_{11}$. The region below *m/z* 500 was diagnostic and similar to that seen for **1**. Fragments for the aglycone were seen at *m/z* 472 (1%) $[C_{30}H_{48}O_4]^+$ and 453 (6%), due to loss of water. In addition, there was a fragment for the diacetyirhamnose unit at *m/z* 246 (7%) which corresponds to $C_{10}H_{,4}O_7$.

Thus **3** was proposed to be a diacetoxy-rhamnoside of hydroxyimberbic acid and was confirmed by ^1H–^1H COSY and HMBC correlations to be 1α,3β,23-trihydroxy-olean-12-en-29-oic acid-23-O-α-[3,4-diacetyl]-rhamnopyranoside. This is the first report of the isolation of this compound.

Compound **4** was isolated as a yellow powder from *C. imberbe*. Its IR spectrum was similar to the other compounds discussed earlier. FABMS was diagnostic of an olean-12-ene-based glycoside with a molecular ion at *m/z* 864 corresponding to $C_{46}H_{72}O_{15}$. Small but significant peaks occurred at *m/z*: 846 $(M-H_2O]^+$(0.6%) and 829 $[M-2H_2O]^+$(0.6%).

The NMR spectra were similar to those of the other compounds discussed above. H-12 appeared as a broad singlet at δ_H 5.14, with H-1, at δ_H 3.52 (*brs*) and, from HSQC, attached to the carbon at δ_C 70.4. H-3 was attached to C-3 at δ_C 67.7 and appeared as a double doublet at δ_H 4.14 ($J=4.5$, 8 Hz) while H-19 was, as with the analogues, split into a well-resolved triplet at δ_H 2.25 and an overlapped multiplet at δ_H 1.78. The sugar region was crowded with duplicated multiplets, with two broad singlets at δ_H 4.74 and 5.94. For ease of discussion and labelling, the rhamnose units are designated Rh′ and Rh″. Using HMQC and ^{13}C spectra, the proton δ_H 4.74 was assigned as the anomeric proton of Rh′ (Rh-1′). ^1H–^1H COSY was used to trace the spin system to Rh-2′ (δ_H 4.07, *s*/δ_C 70.7), Rh-3′ (5.13, *dd*, $J=8/73.1$), Rh-4′ (5.12, *t*, $J=8/73.0$), Rh-5′ (3.88, *m*/67.7) and finally to Rh-6′ (1.27, *s*/18.6). The acetyl

Table 1
HMBC correlations for methyl groups in compound **1**

Position	δ_H	δ_C	^2J	^3J
23	0.97 (*s*)	28.5	40.0 (C-4)	72.7 (C-3), 48.4 (C-5), 17.4 (C-24)
24	1.10(*s*)	17.3	40.0 (C-4)	72.7 (C-3),48.4 (C-5),28.5 (C-23)
25	1.32 (*s*)	16.5	42.5 (C-10)	38.3 (C-9), 71.8 (C-1), 48.4 (C-5)
26	1.07 (*s*)	16.7	39.7 (C-8)	32.8 (C-7), 41.5 (C-14)
27	1.31 (*s*)	26.2	41.5 (C-14)	39.7 (C-8), 144.5 (C-13)
28	1.32 (*s*)	28.9	32.8 (C-22)	27.4 (C-16), 46.7 (C-18), 36.6 (C-22)
30	1.45 (*s*)	20.1	42.8 (C-20)	41.6 (C-19), 29.8 (C-21), 81.4 (C-29)

groups whose methyls resonated at δ_H 2.05 (ca. δ_C 21) were shown to be attached to the Rh-3′/Rh-4′ carbons because these protons were substantially deshielded. This was confirmed from HMBC correlations between the ring protons and the acetoxy carbonyls at ca. δ_C 172.

Table 2
^{13}C NMR spectral data for compounds isolated in this study: data for compounds **1** and **2** are given for comparison

Position	**1**[a,c]	**2**[b,d]	**3**[a,c]	**4**[a,d]	**5**[b,c]
1	72.7	73.0	73.1	73.0	71.7
2	35.8	35.1	35.0	35.0	36.5
3	71.8	67.6	67.8	67.7	66.7
4	40.0	41.2	41.3	41.3	43.0
5	48.4	41.7	42.0	42.0	41.5
6	18.8	19.4	19.5	19.4	18.4
7	32.8	33.4	33.4	33.4	33.2
8	39.7	37.5	41.2	41.3	40.4
9	38.3	39.7	39.6	39.6	38.8
10	42.5	43.7	43.7	43.7	41.5
II	23.7	24.6	24.6	24.6	24.2
12	123.3	124.7	124.6	125.0	123
13	144.5	145.7	145.7	145.5	144.7
14	41.5	42.4	42.4	42.4	43.0
15	26.6	27.8	27.8	27.8	27.2
16	27.4	28.4	28.5	28.4	29.0
17	32.8	33.9	33.9	34.0	33.2
18	46.7	47.9	48.0	47.9	46.8
19	41.6	42.3	42.4	42.2	41.5
20	42.8	43.9	43.9	43.8	43.8
21	29.8	30.8	30.8	30.5	30.8
22	36.6	36.5	37.5	37.3	36.9
23	28.5	71.2	71.7	71.6	71.4
24	17.3	13.1	13.2	13.2	13.5
25	16.5	17.8	17.7	17.8	17.6
26	16.7	18.1	18.1	18.3	17.9
27	26.2	27.2	27.1	27.1	26.7
28	28.9	29.1	29.1	29.1	28.8
29	181.4	183.2	183.2	178.6	177.5
30	20.1	20.2	20.2	20.1	19.9
1′	–	101.9	102.0	102.0	101.8
2′	–	73.0	70.4	70.7	73.2
3′	–	72.3	74.0	73.1	71.0
4′	–	76.1	73.3	73.0	761
5′	–	68.0	68.1	68.1	67.6
6′	–	18.0	18.1	18.2	18.4
3′-OCOCH$_3$	–	–	21.2	21.3	–
3′-OCOCH$_3$	–	–	172.2	172.3	–
4′-OCOCH$_3$	–	21.6	21.3	21.4	21.6
4′-OCOCH$_3$	–	172.9	172.2	172.4	171.2
1″	–	–	–	95.7	96.0
2″	–	–	–	72.6	71.7
3″	–	–	–	73.8	73.0
4″	–	–	–	74.0	72.7
5″	–	–	–	73.8	74.0
6″	–	–	–	18.6	19.1

[a] 125 MHz.
[b] 100 MHz.
[c] C_5D_5N.
[d] CD_3OD.

HMBC data also provided information about the point of glycosylation, showing that R-1′ at δ_H 4.74 coupled to the oxymethine at δ_C 71.6, which was the signal for C-23 on the aglycone and has been shown to be the area of preferred glycosylation in the other compounds discussed earlier. The second sugar could have been attached to positions 1 or 3, or to the carboxylic group at 29. Panzini et al. (1993) have previously isolated bidesmosides of imberbic acid with *O*-glycosyl units at C-3 in addition to C-23. The HMBC spectrum in this case, however, showed Rh-1″ with three-bond correlations to the quaternary carbon at δ_C 178.6 which belonged to the carboxylic acid. It could therefore be concluded that Rh-l″ was attached directly to C-29 to form an ester which was supported by the appearance of a peak in the IR spectrum at ca. 1730 cm^{-1}. Houghton and Lian (1986) have observed that β-linkage through an ester group such as seen here will give an IR peak at about 1726 cm^{-1}.

The additional glycosylation of the aglycone at C-29 resulted in two fundamental changes to the resonances in this area. The anomeric proton was deshielded (δ_C 5.94) while its carbon became shielded (δ_C 95.7) and C-29 moved upfield (δ_C 178.6) by about 3–5 ppm in comparison to the free acid. In general, other resonances of ring E, while not shifting significantly in the carbon domain, showed an upfield shift in the proton domain by between 0.2 and 0.5 ppm. Compound **4** was thus identified as 1α,3β,23-trihydroxy-olean-12-en-29-oate-23-*O*-α[3,4-diacetylrhamnopyranosyl]-29-*O*-α-rhamnopyranoside. This is the first report of this compound.

Compound **5** was isolated as a peach amorphous solid from the stem bark of *T. stuhlmannii*. The FABMS showed pseudomolecular ions at *m/z* 823.2 (6%) $[M+H]^+$ and 846.1 (10%) $[M+H+Na]^+$. These correspond to the molecular formula $C_{44}H_{70}O_{14}$. The region below *m/z* 500 was similar to that of the other olean-12-ene compounds isolated in this study with fragments at *m/z* 472.2 (60%) for $[C_{30}H_{48}O_4]^+$, 453.2 (100%) for $[C_{30}H_{46}O_3]^+$ and those from the retro-Diels–Alder cleavage at *m/z* 248 (34%), 242 (36%) and 236 (50%). This suggested that the aglycone was the same as that of the other triterpenes discussed earlier.

NMR showed that the basic structure of **5** was lα3β,23-trihydroxyimberbic acid, as for the previous compounds. The 1D NMR spectra were almost superimposable on those of **2**, the mono-acetylated rhamnoside. However, they showed subtle changes in the resonances of ring A and ring E with shielding of C-1 (−1 ppm), C-3 (−3.9 ppm) and C-29 (−3.9 ppm) and deshielding of C-4 (+3 ppm) and C-20 (+ 1 ppm), relative to the aglycone. The presence of two anomeric protons showed that there were two sugar units. One of the sugar units was acetylated in position 4 causing both the proton and carbon in this position to resonate at low field (δ_H 5.81 and δ_C 76.1). The two units were

disentangled from each other by ^1H–^1H COSY. The positions of glycosylation were adduced from the chemical shift positions of the anomeric atoms (Shigenaga et al., 1985) and from HMBC correlations.

Compound **5** was thus characterized as 1α, 3β, 3,23-trihydroxy-olean-12-en-29-oate-23-*O*-α-[4-acetoxyrhamnopyranosyl]-29-α-rhamnopyranoside and is reported here for the first time. The isolation of **2** and **5** is significant as it shows that the trihydroxy-olean-12-en-29-oate aglycone, hitherto associated with species of the genus *Combretum,* also exists as a constituent of the genus *Terminalia*. This establishes a chemotaxonomic link between these two genera that has not been previously reported.

Table 3
^1H NMR spectral data for compounds isolated in this study: data for compounds **1** and **2** are given for comparison (values without coupling constant or multiplicity were derived from HMQC)

Position	**1**[a,c]	**2**[b,d]	**3**[a,c]	**4**[a,d]	**5**[b,c]
1	3.86 *brs*	3.33 (*brs*)	3.46 (*brs*)	3.80 (*s*)	3.84 (*brs*)
2	2.19, 2.23	1.31,[e]	1.50, 1.85	1.56, 1.39	1.51, 1.28
3	4.35 (*dd*, *J* = 4.5, 11.5)	3.96 (*dd*, J = 4.5, 12.5)	4.03 (*dd*, *J* = 4.5, 12.5)	4.14 (*dd*, J = 4.5, 12.5)	4.96 (*dd*, *J* = 5, 12)
4	–	–	–	–	–
5	1.75	s	1.71	1.67	1.49
6	1.72, 1.35	1.30, 1.22	1.41, [e]	1.56, [e]	1.68, [e]
7	1.60, 1.36	1.75, 1.52	1.24, 1.26	1.56, 1.35	1.88, 1.41
8	–	–	–	–	–
9	2.88 (*m*)	2.32 (*m*)	2.45 (*m*)	2.55 (*m*)	3.01 (*m*)
10	–	–	–	–	–
11	2.02, 2.31	1.68, [e]	1.85	1.98, 1.97	2.32, 2.06
12	5.36 (*brs*)	5.07 (*brs*)	5.17 (*brs*)	5.29 (*brs*)	5.35
13	–	–	–	–	–
14	–	–	–	–	–
15	1.85, 1.05	1.54, 0.84	0.95, 1.70	1.76, 1.78	1.52, 1.90
16	0.87, 2.13	1.88, 0.81	0.92, 1.99	2.08, 2.11	2.05, 1.51
17	–	–	–	–	–
18	2.16	1.84,	1.94	2.05	2.10
19	1.71, 2.50 (*t*, *J* = 13.5)	2.04 (*t*, *J* = 13.5), 1.30	1.21, 2.14 (*t*, *J* = 13.5)	2.25 (*t*, *J* = 14), 1.39[e]	2.44 (*J* = 13.5), 2.33
20	–	–	–	–	–
21	2.20, 1.72	1.58, 1.22	1.24, 1.72	1.90, 1.47	[e]
22	1.53, 1.5	1.14, 1.10	1.20, 1.43	1.56, 1.39	[e]
23	0.97 (*s*)	3.32, 3.06 (ABq, *J* = 9)	3.33, 3.53 (ABq *J* = 9.5)	3.37, 3.30 (ABq, *J* = 9.5)	3.95, 3,84 (ABq, *J* = 9)
24	1.10 (*s*)	0.52 (*s*)	0.73 (*s*)	0.74 (*s*)	1.09 (*s*)
25	1.32 (*s*)	0.81 (*s*)	1.02 (*s*)	1.03 (*s*)	1.14 (*s*)
26	1.07 (*s*)	0.85(*s*)	1.05(*s*)	1.16 (*s*)	1.16(*s*)
27	1.31 (*s*)	1.08 (*s*)	1.27 (*s*)	1.28 (*s*)	1.39 (*s*)
28	1.32 (*s*)	0.69 (*s*)	0.89 (*s*)	1.03(*s*)	0.98 (*s*)
29	–	–	–	–	
30	1.45 (*s*)	1.01 (*s*)	1.22 (*s*)	2.05	1.31 (*s*)
1′	–	4.50 (*s*)	4.63 (*s*)	4.74 (*s*)	5.22 (*brs*)
2′	–	3.71 (*s*)	3.96 (*brs*)	4.07 (*s*)	4.42 (*brm*)
3′	–	3.73 (*m*)	5.07 (*dd*, *J* = 3,10)	5.18 (*dd*, *J* = 3,10)	4.62 (*dd*, *J* = 3.2, 9.4)
4′	–	4.74 (*t*, *J* = 9.5)	5.02 (*t*, *J* = 10)	5.12 (*t*, *J* = 10)	5.81 (*t*, *J* = 9.7)
5′	–	3.58 (*m*)	3.78 (*m*)	3.84 (*brm*)	4.27 (*brm*)
6′	–	0.95 (*d*, *J* = 6)	1.19 (*d*, *J* = 6)	1.20 (*d*, *J* = 5.5)	1.45 (*d*, *J* = 6.2)
3′-OAc	–	–	2.05 (*s*)	2.05 (*s*)	–
4-OAc	–	1.88 (*s*)	2.05 (*s*)	2.05 (*s*)	2.06 (*s*)
1″	–	–	–	5.94 (*s*)	6.79 (*brs*)
2″	–	–	–	3.68 (*brs*)	4.54 (*m*)
3″	–	–	–	3.57 ([e])	4.52 (*dd*, *J* = 3.3, 5.6)
4″	–	–	–	3.46 (*t*, *J* = 9.5)	4.33 (*t*, [e])
5″	–	–	–	3.67 (*m*)	4.31 (*brm*)
6″	–	–	–	1.27 [e]	1.62 (*d*, *J* = 5.8)

[a] 500 MHz.
[b] 400 MHz.
[c] C_5D_5N.
[d] CD_3OD.
[e] Designates poorly resolved chemical shifts and/or coupling constants, *J* is coupling constant in Hz.

2.1. Biological activity

A microtitre dilution assay (MDA) was used to screen for antimicrobial activity of the isolated compounds (Drummond and Waigh, 2000). The results of all the compounds tested are presented in Table 4.

Compound **4** showed inhibitory activity against *P. vulgaris* (12.5 μg/ml) and *Staphylococcus aureus* (6.25 μg/ml), while **5** acted against *Candida albicans* (12.5 μg/ml) and *S. aureus* to a lesser extent (25 μg/ml). Compound **1**, the free aglycone, showed activity against *Mycobacterium fortuitum* at a concentration of 1.56 μg/ml and *S. aureus* at 3.13 μg/m1. This was the more surprising considering that the *Mycobacterium* was generally resistant to the other test samples. Compound **2** inhibited *S. aureus* at 12.5 μg/ml while **3** inhibited *S. aureus* at 6.25 μg/ml and *M. fortuitum* at 12.5 μg/ml.

It was noted that *Escherichia coli* was resistant to these compounds, suggesting that these constituents of these species of Combretaceae may not be active against gram negative bacteria. The pentacyclic and tetracyclic triterpenes are best known for their action as molluscides particularly in their monodesmosidic form (Msonthi et al., 1995; Marston and Hostettmann, 1985). In this regard, *Combretum molle* is best known for its molluscidal constituent, mollic acid, which has been recommended for use in rural Africa to control schistosomiasis (Rogers, 1995). Apart from this work, there are few data on the biological potential of isoprenoid constituents of Combretaceae. Martini and Eloff (1998) have gathered preliminary data to show that crude extracts of *C. eryththrophvllum* are active against microbial cultures which may support their use in traditional medicine for relieving symptoms that appear to be caused by infective agents e.g. bloody diarrhoea (possibly cholera and dysentery), wounds and conjunctivitis (Gelfand et al., 1985).

The present results may justify the use of these plants in folk medicine. These compounds are candidates for further work to evaluate their therapeutic potential.

Table 4
Minimum inhibitory concentrations for isolated compounds (μg/ml)

Compound	CA	EC	MF	PV	SA
1	100	>100	1.56	25	3.13
2	>100	>100	>100	25	12.5
3	50	>100	12.5	25	6.25
4	>100	>100	25	12.5	6.25
5	12.5	>100	25	100	25
Flu	12.5				
Strep		3.12	1.56	3.12	0.78
DMSO	>100	>100	>100	>100	>100

CA, *Candida albicans*; EC, *Escherichia coli*; MF, *Mycobacterium fortuitum*; PV, *Proteus vulgaris*; SA, *Staphylococcus aureus*; Flu, fluconazole; Strep, streptomycin.

3. Experimental

3.1. General

NMR spectra were recorded with either a Brüker DRX500 NMR spectrometer equipped with an autotune probe and using the automation mode aided by the Brüker programme, Icon-NMR, or a Brüker AMX 400MHz NMR instrument. Infra-red spectra were recorded on a Matson Genesis series FT-IR spectrophotometer as KBr discs or by drying trichloromethane solutions of the samples on NaCl windows. Melting point (mp) (uncorrected) measurements were made using the Reichert Apparatus (Austria). HREIMS were run on a Jeol JMS-AX505HA double focusing instrument at 70 eV. FABMS were also run on this instrument using glycerol as the matrix.

In all cases the preparative HPLC method used was a gradient of water/acetonitrile with 0.1% trifluoroacetic acid (TFA) (90%/10% to 0%/100%) for 20–30 min on Waters LC with UV detector. PrepLC column 40 mm (i.d.) and 100 mm long were used with Merck Anal reagents as eluents.

Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide) (Sigma, UK) was used to make the indicator solution for the microdilution assay. This dye is purple/pink when cells are viable and blue where cells are not viable.

3.2. Plant material

The leaves of *C. imberbe* and the stem bark of *T. stuhlmannii* were collected from a peasant plot at Mahuwe, Zambezi Valley in Northern Zimbabwe in May l999. A voucher specimen has been deposited at the herbarium of the Harare Botanical Gardens and at Edinburgh Botanical Gardens, UK.

3.3. Extraction and isolation

Dry leaf material of *C. imberbe* (762.0 g) was collected and ground, of which 211.5 g was defatted with n-hexane and extracted with DCM at room temperature by maceration. A yield of 17.6 g was obtained which was added in solution to Dialon HP20 resin powder and dried on a rotary evaporator until the resin was free flowing. The resin was packed into a Buchner funnel and eluted with 10% acetone in methanol under vacuum. The eluent was dried using a rotary evaporator and the material obtained (3.9 g) fractionated by gel filtration on Sephadex LH-20, using first 100% TCM and then increasing concentrations of methanol. The collected fractions were further fractionated by preparative HPLC, resulting in the isolation of four triterpenoids. These were **1** (70 mg, $t_R = 8.4$ mm), **2** (36.3 mg, $t_R = 22.5$ mm), **3** (25.3 mg, $t_R = 25.5$ mm) and **4** (47.4 mg, $t_R = 20$ mm).

The dry stem bark of *T. stuhlmannii* (751.6 g) was defatted with *n*-hexane and extracted by maceration with DCM yielding 826.6 mg. This gave 632.4 mg of material after treatment with HP-20, as described earlier, which was loaded onto a Sephadex LH-20 column. The fractions collected from the gel filtration column were further fractionated by preparative HPLC. Four compounds were detected, but only **2** and **5** (7.6 mg, $t_R = 16$ mm) were isolated in pure form.

3.4. Microdilution assay (MDA)

The indicator solution (resazurin) was prepared by adding one tablet to 40 ml of sterile water. The test organism was prepared by diluting overnight broth culture with sterile diluent (0.1% Peptone water). The turbidity was adjusted to match 0.5 McFarland standard. The broth culture was then diluted 100-fold to give a concentration 10^8 cfu/ml. The test sample was made up as a stock solution of 200 μg/ml solubilized with less than 20 μl of DMSO in sterile water.

A mixture of indicator and broth medium was prepared by adding 1 ml of indicator solution to 7.5 ml of broth medium. One hundred microlitres of this mixture was pipetted into sterility control wells in the 96-well plate. Five millilitres of the broth culture was then added to the mixture of indicator and broth medium made earlier and 100 μl of this pipetted into test and growth control wells in plate. The test solution (100 μl) was pipetted into well one of the plate, mixed well and 100 μl was transferred into well two. This mixing and transferring was repeated until well and then the pipette tip was discarded with its contents.

The plates were incubated at 31 °C for at least 5 h and taken out at the time the dye changed colour for the first time to pink. The test wells were read high to low concentration and compared with the sterility and growth controls. The concentration in the last well showing no colour change (blue) was taken as the minimum inhibitory concentration (MIC).

The organisms used in this study and their incubation times were: *C. albicans* 8–24 h, *E. coli* 8 h, *M. fortuitum* 24–48 h, *P. vulgaris* 8 h, *S. aureus* 8 h.

3.4.1. 1α,3, β-Hydroxyimberbic acid (1α,3, β-dihydroxy-olean-12-en-29-oic acid) (*1*)

White to colourless crystalline solid, mp 282 °C (286–288 °C, Rogers and Subramony, 1988) $[\alpha]_D^{25}$ +41.7° (*c* 0.3, MeOH). IR ν_{max} KBr cm^{-1}: 3437, 2949, 1697, 1647, 146, 1381, 1225, 1033, 915, 736. Found $[M+H]^4$ 473.3635. ($C_{30}H_{48}O_4$ requires 472.3553). HRFABMS *m*/*z* (rel. in.%): 472.30 (3) 453.40 (19), 436.33 (6), 395 (12), 248.2 (10), 188.9 (100). 129.6 (40), 93.1 (74). ^1H and ^{13}C NMR Tables 2 and 3.

3.4.2. 1α,3, β-Hydroxyimberbic acid-23-O-α-L-4-acetyl-rhamnopyranoside (1α,3, β,23-trihvdroxy-olean-12-en-29-oate-23-O-α-L-4-acetylrhamnopyranoside) (*2*)

White amorphous solid, mp 198 °C (Lit. 235–238). $[\alpha]_D^{21}$ +41.8° (*c* 0.311, MeOH). Found $[M+Na]^+$ 701.4 and $[M+H]^+$ 677.4. ($C_{38}H_{61}O_{10}$ requires 677.4). HRFABMS *m*/*z* (rel. int.%): 661.439 (19), 658.418 (41), 472.0 (18), 453.5 (80), 437.0 (30), 395.5 (50), 248.3 (100), 231.2 (62). ^1H and ^{13}C NMR Tables 2 and 3.

3.4.3. 1α,3, β-Hydroximberbic acid-23-O-α-L-3,4-di-acetylrhamnopyranoside (1α,3, β-trihydroxy-olean-12-en-29-oate-23-O-α-L-3, 4-acetylrhamnopyranoside) (*3*)

Yellow amorphous solid, mp 190 °C. $[\alpha]_D^{25}$ +56.8° (*c* 0.22, MeOH). IR ν_{max} KBr cm^{-1}: 3446, 2944, 1732, 1454, 1378, 1240, 1127, 1075, 820, 603. Found $[M+H]^+$ 719.4289. ($C_{40}H_{62}O_{11}$ requires 718.4292). LRFABMS *m*/*z*: (rel. int.%): 719.4 (0.4), 701.1 (1), 683.1 (1), 659 (0.2), 641.2 (0.2), 472.0 (1.5), 453.2 (5.5), 437 (3.5), 395.2 (3.5), 261.1 (2.5), 48 (80) 231.1 (100). ^1H and ^{13}C NMR Tables 2 and 3.

3.4.4. 1α,3, β-Hydroxyimberbic acid-23-α-[L-3, 4-diacetyl- rhamnopyranosyl]-29-O-α-rhamnopyranoside (1α,3, β,23-trihydroxy-olean-12-en-29-oate-23-O-α-L-3, 4-acetyl-29-dirhamnopyranoside) (*4*)

Yellow amorphous solid, mp 178 °C. $[\alpha]_D^{22}$ +16.2° (*c* 0.401, MeOH). IR ν_{max} KBr cm^{-1}: 3446, 2939, 1732, 1455, 1382, 1217, 1047, 965, 799, 603. Found M^+ 864.487 ($C_{46}H_{72}O_{15}$ requires 864.487). LRFABMS *m*/*z* (rel. int.%): 864.4 (0.4), 846.1 (0.6), 828.0 (0.3), 769.1 (0.3), 701.3 (3), 683.2 (3), 599.0 (1), 472.0 (2), 453.1 (9), 436.0 (5.5). ^1H and ^{13}C NMR Tables 2 and 3.

3.4.5. 1α,3, β-Hydroxyimberbic acid-23-O-α-[L-4-acetyl- rhamnopyranosyl]-29-O-α-rhamnopyranoside (1α,3, β,23-trihydroxy-olean-12-en-29-oate-23-O-α-L-4-acetyl-29-dirhamnopyranoside) (*5*)

Peach amorphous solid, mp 196 °C. $[\alpha]_D^{22}$ +15.0° (*c* 0.133, MeOH). Found $[M+H]^+$ 823.1 ($C^{44}H_{71}O_{14}$ requires 823.5). LRFABMS *m*/*z* (rel. int.%): 823.1 (8), 804.5 (7), 752.6 (8), 677.4 (10), 659.1 (21), 641.2 (21), 472.0 (30), 453.2 (100), 395.3 (60), 335.1 (50), 248.0 (34), 242 (36), 201.1 (65). ^1H and ^{13}C NMR Tables 2 and 3.

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