

Antimicrobial and anti-inflammatory activity of four known and one new triterpenoid from *Combretum imberbe* (Combretaceae)

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Abstract

Combretum imberbe is used widely in Africa *inter alia* for treating bacterial infections. In addition to four known triterpenoids, 1 α ,3 β -dihydroxy-12-oleanen-29-oic (**1**), 1-hydroxy-12-olean-30-oic acid (**2**), 3,30-dihydroxyl-12-oleanen-22-one (**3**), and 1,3,24-trihydroxyl-12-olean-29-oic acid (**4**), a new pentacyclic triterpenoid (1 α ,23-dihydroxy-12-oleanen-29-oic acid-3 β -O-2,4-di-acetyl-l-rhamnopyranoside) **5** has been isolated through a bioassay-guided procedure from the leaves of *Combretum imberbe*. The structures of the compounds were elucidated on the basis of 1D and 2D NMR experiments, as well as mass spectrometric data. All compounds isolated had moderate (62 $\mu\text{g/ml}$) to strong (16 $\mu\text{g/ml}$) antibacterial activity (MIC values) against *Staphylococcus aureus* and *Escherichia coli*, with **1** and **5** being most active. Compounds **1** and **5** also showed very strong inhibition of 3 α -hydroxysteroid dehydrogenase with an IC₅₀ of 0.3 $\mu\text{g/ml}$. Compound **5** indicated a moderate anti-proliferative (GI₅₀ = 16.5 and 13.2 $\mu\text{g/ml}$) and cytotoxic activity (CC₅₀ = 17.6 $\mu\text{g/ml}$) against K-562, L-929 and HeLa cell lines, respectively. The results of this study give credence to the ethnomedicinal use of *Combretum imberbe* and expand our knowledge on the biological activity of its metabolites.

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1. Introduction

Combretaceae is a large family with at least 600 species. *Combretum* is one of the two most commonly occurring genera and widely used in African traditional medicine. The leaves of *Combretum* species are widely used for treating abdominal disorders (e.g. abdominal pains, diarrhea) backache, bilharziasis, chest coughs, colds, conjunctivitis, dysmenorrhoea, earache, fattening babies, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion and snake bite, swelling caused by mumps, syphilis, toothache and general weakness (Oliver-Bever, 1986 and Hutching et al., 1996). Preliminary work indicated that *Combretum imberbe* contains several antibacterial compounds (Eloff, 1999). Rogers and Subramony (1988) have isolated pentacyclic triterpenes acids from the leaves of this species, but did not investigate the biological activity. Rogers and Subramony (1988), as well as Katerere et al. (2003), have isolated related glycosides all based on the olean-12-29-oate aglycone (imberbic acid) from this

plant. Earlier studies (Katerere et al., 2003) have shown that pentacyclic triterpenes from *Combretum imberbe* have antibacterial activity against *Mycobacterium fortuitum*. Despite the substantial phytochemical work reported on *Combretum imberbe*, the biological activities of compounds isolated from this plant have not been examined to a large extent for other activities that may be related to ethnomedicinal use. This paper also reports on anti-inflammatory, anti-proliferative, cytotoxic, as well as antibacterial activities of one new (**5**) and four known (**1–4**) pentacyclic triterpenes from *Combretum imberbe*.

2. Materials and methods

2.1. General experimental procedures

Column chromatography, silica gel 60M (230–400 mesh, Machery-Nagel, Germany), Sephadex LH-20 (Pharmacia Biotech AB, Sweden); TLC, silica gel plates (Sil G/UV₂₅₄, 0.20 mm, Machery-Nagel, Germany), spots were detected under a UV lamp and sprayed with vanillin/H₂SO₄ spray reagent. Optical rotation, Propol digital automatic polarimeter (Dr. Wolfgang Kernchen GMBH, Germany); IR spectra, IFS55 spectrometer (Bruker, Germany); ¹H and ¹³C NMR spectra, DPX-300, DNMR, DPX-500 (Bruker, Germany), measured in CD₃OD or CDCl₃ with reference against TMS (external); ESIMS, triple quadrupole mass spectrometer Quattro (VG Biotech, England); EIMS, 70 eV direct inlet, high resolution with perfluorokerosine as a standard, MAT 95 XL (Finnegan, Germany).

2.2. Plant material

The leaves of *Combretum imberbe* were collected from National Botanical Garden in Nelspruit, South Africa in September 2002. The tree was identified by the curator for Combretaceae Mr Johan Hurter and by the tree label indicating origin and date collected. A voucher specimen is deposited in the Lowveld National Botanical Garden Herbarium, Nelspruit, South Africa.

2.3. Extraction and isolation

Air-dried and powdered leaves (500 g) of *Combretum imberbe* were defatted with hexane and extracted serially and exhaustively by maceration with dichloromethane (DCM),

acetone and methanol (MeOH) to afford 18.2, 16.5, 25.5 and 41.5 g of *n*-hexane, DCM, acetone and MeOH soluble extracts, respectively. The DCM (16.5 g) extract was the most active extract (MIC 39 µg/ml against *Staphylococcus aureus*) was subjected to liquid–liquid fractionation (Eloff, 1998a) that afforded six fractions [chloroform (6.5 g), water (0.125 g), *n*-butanol (0.476 g), hexane (0.8), carbon tetrachloride (1.356 g), 35% H₂O in MeOH (0.450 g)]. The chloroform fraction from the DCM extract (6.5 g), had an excellent antibacterial activity (MIC 10 µg/ml) was chromatographed on a 2 cm × 30 cm silica gel 60 open column using a stepwise gradient of *n*-hexane and increasing amounts of ethyl acetate (EtOAc) (20% and 800 ml each step), followed by EtOAc with increasing amounts of MeOH (10% and 800 ml each step, ending at 40%). Collected fractions were evaporated under vacuum and examined by TLC. Fractions with a similar composition were pooled to give 12 major fractions (*F*₁–*F*₁₂) that all had antibacterial activity. All tubes were filled with EtOAc and allowed to evaporate under a stream of cool air. Crystals were collected from *F*₂ and *F*₆ and washed with hexane to give 80 mg of **1** and 7 mg of **2**, respectively. *F*₁ (0.78 g) was run on a 1 cm × 20 cm silica gel 60 column to yield 7 mg of **3**. *F*₉–*F*₁₂ were further combined, dried by rotary evaporator to yield *F*₁₃ (3.2 g). *F*₁₃ was subjected to Sephadex LH20 (3 cm × 120 cm, MeOH) to yield six sub-fractions *F*_{13.1}–*F*_{13.6}. Sub-fractions *F*_{13.4} and *F*_{13.5} were combined (0.3 g) and subjected to silica gel 60 2 cm × 30 cm column eluted with chloroform/methanol (9:1 1200 ml) to yield **4** (8 mg) and **5** (6 mg).

2.4. Biological assays

2.4.1. Anti-microbial assays

The microplate serial dilution method (Eloff, 1998b) was used to determine the minimum inhibitory concentration (MIC) of fractions and pure compounds against *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922. Extracts (10 mg/ml) or isolated compounds (1 mg/ml) were dissolved in acetone and serially diluted with sterile water in microplates in a laminar flow cabinet. The same volume of an actively growing culture of the test bacteria was added to the different wells and cultures were grown overnight in 100% relative humidity at 37 °C. The next morning tetrazolium violet was added to all

the wells. Growth was indicated by a violet colour of the culture. The lowest concentration of the test solution that led to an inhibition of growth was taken as the MIC. The negative control acetone had no influence on the growth at the highest concentration used (25%).

2.4.2. Bioautography

Bioautography (Begue and Kline, 1972) was used in the bioassay-guided isolation of antibacterial compounds and fractions. Developed chromatography plates (5 μ l of 10 mg/ml = 50 μ g) of extracts and fractions were dried over night and sprayed with a suspension of growing cells of the bacteria listed above and incubated at 37 °C in a chamber at 100% relative humidity for 18 h. After spraying with tetrazolium violet, clear zones on the chromatogram indicated inhibition of growth after incubating for 1 h at 37 °C.

2.4.3. Anti-inflammatory activity assay against 3 α -hydroxysteroid dehydrogenase

The assay on the inhibition of 3 α -hydroxysteroid dehydrogenase from rat liver cytosol was as according to Penning (1985). Increasing amounts of isolated compounds were added to the standard assay system, and the concentration of compound required to reduce the rate of 5 β -dihydrocortisone reduction by 50% (IC₅₀ value) was determined from the resulting dose–response curves.

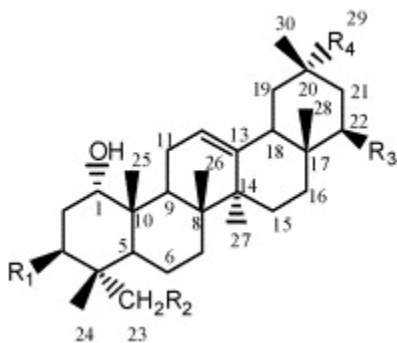
2.4.4. Cytotoxic and anti-proliferative efficacy

Isolated compounds were assayed on K-562 (DSM ACC 10) and L-929 (DSM ACC 2) cell line for their anti-proliferative and against HeLa (DSM ACC 57) for their cytotoxic activity as described by Dahse et al. (2000).

3. Results and discussion

After exhaustive extraction of the dried and powdered leaves of *Combretum imberbe* (500 g) with hexane, dichloromethane, acetone and methanol, bioautography against *Staphylococcus aureus* indicated that the highest number of antibacterial compounds were present in the DCM extract. Bioautography and MIC results indicated that the

chloroform fraction after solvent/solvent fractionation of the DCM extract was the most active fraction (MIC 10 $\mu\text{g/ml}$) with the highest number of antibacterial compounds. Exhaustive gradient column chromatography of the active chloroform fraction yielded twelve fractions that were all active against *Staphylococcus aureus* according to bioautography. Further chromatography of the fractions on silica gel 60 and Sephadex LH20 columns yielded five oleanene-type triterpenoids (**1–5**).



Compounds	R ₁	R ₂	R ₃	R ₄
1	OH	H	H	COOH
2	H	H	H	COOH
3	H	H	=O	CH ₂ OH
4	H	H	OH	COOH
5	2,4-Di-Ac-Rh	OH	H	COOH

IR spectra indicated the presence of olefinic (1650 cm^{-1}) and hydroxyl (3440 cm^{-1}) moieties in **1–5** and a carboxylic acid (1680 cm^{-1}) in **1**, **2**, **4** and **5**. ^{13}C NMR spectra of **1** suggested an oleanane skeleton with a carboxylic function, a trisubstituted double bond, two carbons bearing OH groups and an AB system as has been previously described from this species (Katerere et al., 2003). This pattern, along with similar ^1H NMR and mass spectrometric data, was also observed in compounds **2–4**, suggesting a similar skeleton for all four compounds. The only difference between these three molecules could be observed in ring A and E. According to a perfect match of the experimental data with the

literature, they were identified as 1 α ,3 β -dihydroxy-12-oleanen-29-oic (**1**) (Katerere et al., 2003 and Rogers and Subramony, 1988), 1-hydroxy-12-olean-30-oic acid (**2**) (Mukherjee et al., 1994), 3,30-dihydroxyl-12-oleanen-22-one (**3**) (De Sousa et al., 1990) and 1,3,24-trihydroxyl-12-olean-29-oic acid (**4**) (Duan and Takaihi, 2001), respectively. Compound **1** (imberbic acid) crystallized in most of the fractions and was isolated in large quantity (80 mg). It has been previously isolated from the same species and appeared to be the major constituent in the leaves of *Combretum imberbe*. Compounds **2**, **3** and **4** have been isolated from different plant species, but no biological activities were reported on them. Compound **5**, a glycoside, was isolated and reported here for the first time.

Compound **5** was isolated as white crystals and has a molecular formula of C₄₀H₆₂O₁₁ as determined by ¹H, ¹³C NMR, DEPT data and HRESI data of a [M + NH₄]⁺ ion peak (*m/z* 736.4613, calcd. 736, 4636). The IR spectrum indicated similar structural features as in compounds **1–4**. ¹H NMR signals at δ_H 5.25 indicated a vinylic (R₂C = CHR) moiety and those at δ_H 3.6 and 3.7 hydroxylated methines in the aglycone. The signals at δ_H 4.70 and δ_C 98.62 indicated an anomeric CH group (acetal) of an oxygen-linked sugar moiety. The ¹³C NMR data of **5** (Table 1) were almost identical to those of 1 α ,3 β ,23-trihydroxy-12-oleanen-29-oic acid (Rogers and Subramony, 1988) except for an additional sugar moiety and the missing hydroxyl group at C-3 that had been replaced with the ether linked sugar moiety, as indicated by δ_C 67.0. The location of the new sugar moiety was confirmed by HMBC correlations between H-3 and the anomeric carbon and between the anomeric proton and C-3. Two acetylated methines were indicated at δ_C 75.4, δ_H 4.90 (C-4') and δ_C 73.9, δ_H 5.11 (C-2') through correlations to two acetyl groups. All ¹H and ¹³C NMR data were assigned through HMQC, HMBC and COSY experiments.

Table 1.

¹H (300 MHz) and ¹³C (75.4 MHz) NMR data of **5** in CD₃OD

Position	¹³ C (δ)	¹ H (δ , mult., <i>J</i> in Hz)
C-1 CH	72.6	3.55 t br
C-2 CH ₂	34.6	1.74 m; 2.0, m
C-3 CH	67.0	4.17 dd (9.8, 4.5)
C-4 C	43.2	–

Position	^{13}C (δ)	^1H (δ , mult., J in Hz)
C-5 CH	41.3	1.75 m
C-6 CH ₂	18.9	1.45 m, 1.54 m
C-7 CH ₂	33.0	1.30 m, 1.60 m
C-8 C	40.7	–
C-9 CH	39.2	2.52 dd (9.3, 9.3)
C-10 C	41.8	–
C-11 CH ₂	24.1	1.95 m
C-12 CH	124.3	5.25 s
C-13 C	145.3	–
C-14 C	43.5	–
C-15 CH ₂	27.4	1.86 m
C-16 CH ₂	27.9	2.05 m
C-17 C	33.5	–
C-18 CH	47.5	2.05 m
C-19 CH ₂	41.9	C-1' CH
C-20 C	43.6	C-2' CH
C-21 CH ₂	30.4	C-3' CH
C-22 CH ₂	37.0	C-4' CH
C-23 CH ₃	12.6	C-5' CH
C-24 CH ₂	70.6	C-6' CH ₃
C-25 CH ₃	17.3	C-1'' C=O
C-26 CH ₃	17.7	C-2'' CH ₃
C-27 CH ₃	26.8	C-3'' C=O
C-28 CH ₃	28.6	C-4'' CH ₃
C-29 CH ₃	19.7	1.20 s

Position	¹³ C (δ)	¹ H (δ, mult., <i>J</i> in Hz)
C-30 C	182.8	–
C-1' CH	98.6	4.60 s br
C-2' CH	73.9	5.11 m
C-3' CH	68.8	4.11 dd (3.5, 9.8)
C-4' CH	75.4	4.90, dd (9.8, 9.8)
C-5' CH	67.6	3.81 m
C-6' CH ₃	17.6	1.14 d (6.4)
C-1'' C=O	172.2	–
C-2'' CH ₃	21.0	2.12 s
C-3'' C=O	172.2	–
C-4'' CH ₃	20.8	2.08 s

δ values are expressed in ppm; ¹H (CD₃OD, 300 MHz); ¹³C NMR (CD₃OD, 75.4 MHz).

According to the coupling constants and correlation peaks in NOESY spectrum, **5** had the same relative stereochemistry in the triterpenoid skeleton as 1α,24β-dihydroxy-12-oleanen-29-oic acid. The relative configuration of the chiral centres at C-1, C-3, C-4, C-8, C-15, C-20 and C-10 was determined by detailed NOESY analysis (Fig. 1). The rhamnose was determined to be attached as β anomer on the basis of the ¹³C NMR low field chemical shift of the anomeric carbon (δ_C 98.6) and thus, together with biogenetic grounds, is assumed to be the l-isomer (Seidel, 1999 and Rogers, 1989). Altogether, compound **5** was elucidated as 1α,23-dihydroxy-12-oleanen-29-oic acid-3β-*O*-2,4 di-acetyl-l-rhamnopyranoside. This compound is reported here for the first time [white crystalline solid, [α]_D²⁶ + 36.6° (CH₃OH, *c* 0.31). ESIMS: *m/z* 718 [*M* + Na]⁺. IR (KBr pellet) ν_{max} (cm⁻¹) 3437, 2949, 1725, 1647, 1381, 1225, 1033, 915, 736. NMR data see Table 1].

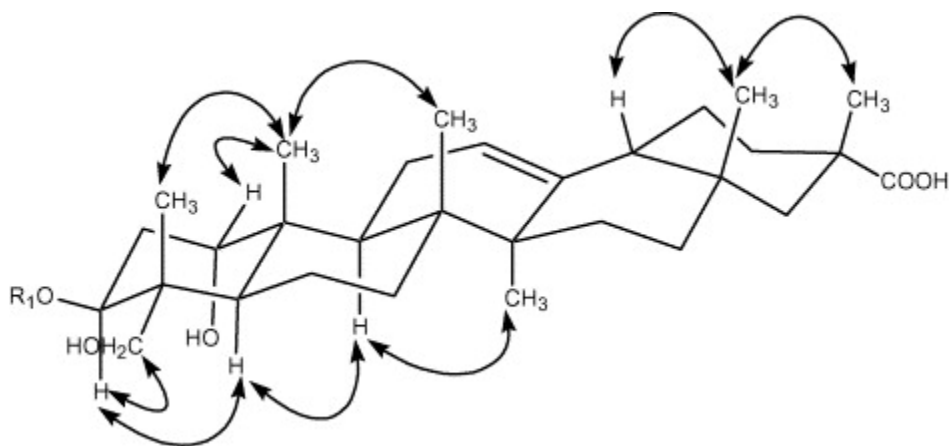


Fig. 1. NOE correlations in compound **5** as detected by NOESY data.

Regarding biological activity, pentacyclic and tetracyclic triterpenes are known for their action as molluscides (Marston and Hostettmann, 1985). *Combretum molle* with its molluscidal constituent, mollic acid, has been recommended for use in rural Africa to control schistosomiasis (Rogers, 1995). Arjunolic acid and arjungenin, arjunglucoside pentacyclic triterpenes have been isolated from *Combretum molle* (Panzini et al., 1993). All compounds isolated in this study were subjected to several assays for bioactivity profiling.

A serial microplate dilution assay (Eloff, 1998b) was used to screen for the antibacterial activity of the isolated compounds (Table 2). Compounds **1**, **3**, **4**, and **5** had a similar activity (16 µg/ml) against *Escherichia coli*. This was despite the fact that *Escherichia coli* was resistant to oleanene type pentacyclic triterpenes isolated from the same plant species by Katerere et al. (2003). Against *Staphylococcus aureus*, **2** and **3** had an MIC of 93 µg/ml compared to 63 µg/ml for both **4** and **5**. The latter two compounds had hardly any activity against *Enterococcus faecalis* and *P. aeruginosa*. Compounds **4** and **5** might be the major antibacterial compounds against *Escherichia coli* and *Staphylococcus aureus* present in *Combretum imberbe* in consideration of their MIC values and bioautogram observation. The activity of the DCM extract (39 µg/ml) against *Staphylococcus aureus* was lower than that of the major compounds isolated. This may have resulted from synergistic effects of different antibacterial compounds or of antibacterial compounds and other compounds in the extract. Apart from our results there is limited data on the anti-microbial potential of the isoprenoid constituents of Combretaceae. Eloff (1999) had gathered preliminary data to indicate that crude extracts

of *Combretum imberbe* and *Combretum padoides* are active against four nosocomial bacteria. Crude extracts of *Combretum erythrophyllum* are active against microbial cultures and contain up to 14 different antibacterial compounds (Martini and Eloff, 1998). Seven of these compounds were isolated and characterized as flavonoids with several biological activities (Martini et al., 2004a and Martini et al., 2004b). The activity of crude extracts support the use in traditional medicine for diseases caused by infective agents, e.g. bloody diarrhoea, wounds and conjunctivitis (Gelfand et al., 1985).

Table 2.

Minimum inhibitory concentration (MIC) in $\mu\text{g/ml}$ of compounds **1–5** towards different pathogens [SA: *Staphylococcus aureus* (ATCC 29213); PA: *Pseudomonas aeruginosa* (ATCC 27853); EC: *Escherichia coli* (ATCC 25922); EF: *Enterococcus faecalis* (ATCC 29212)]

Compounds	SA	EF	PA	EC
1	125	125	>250	16
2	94 [44]	24 [11]	>250	>250
3	125	125	>250	16
4	63	>250	>250	16
5	63	>250	>250	16
Gentamycin	5.8	5.8	5.8	5.8
Acetone	>25%	>25%	>25%	>25%

Standard deviation 0 except where indicated in brackets, cut off point 250 $\mu\text{g/ml}$ for compounds and 25% for solvent negative control.

The anti-proliferative and cytotoxic efficacy of **1**, **3**, **4** and **5** was tested *in vitro* against L-929, K-562 and HeLa cell lines, respectively (Table 3). Compound **3** had a strong and **5** a moderate activity against all three cell lines. In the testing on inhibition of 3α -hydroxysteroid dehydrogenase (Penning, 1985), **1** and **3** showed a strong activity (IC_{50} of 0.30 $\mu\text{g/ml}$ each), whereas **4** (>50 $\mu\text{g/ml}$ each) and **5** (16.5 and 13.5 $\mu\text{g/ml}$).

Table 3.

Cytotoxic and anti-proliferative activity of 1,3-5 with different cell lines

Compounds	Anti-proliferative activity		Cytotoxicity
	L-929 GI ₅₀ (µg/ml)	K-562 GI ₅₀ (µg/ml)	HeLa CC ₅₀ (µg/ml)
1	32.9	28.1	34.9
3	9.0	8.7	10.5
4	>50	>50	47.3
5	16.5	13.5	17.5
Taxol	0.1	0.01	0.01

4. Conclusion

Triterpenoids of *Combretum imberbe* leaves had a reasonably antibacterial activity on *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*. It appears that the major antibacterial compounds were isolated, but the antibacterial activity of the crude extract was higher than could be extrapolated from the activity of the isolated compounds. This points to synergistic effects and justifies the use of *Combretum imberbe* extracts against bacterial infections. Several *Combretum* species had reasonable anti-inflammatory activity. The inhibition of 3 α -hydroxysteroid dehydrogenase activity of some of the isolated compounds confirms the tentative conclusion that the same compounds in *Combretum* species may be responsible for antibacterial and anti-inflammatory activity (Eloff et al., 2001).

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