Clerodendrumic Acid, a New Triterpenoid from *Clerodendrum glabrum* (Verbenaceae), and Antimicrobial Activities of Fractions and Constituents

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One new triterpenoid, (3β,11α,19β)-3-(butanoyloxy)-11-hydroxytaraxast-20(30)-ene-23,28-dioic acid (clerodendrumic acid; **1**) was isolated from the hexane extract of the leaves of *Clerodendrum glabrum* var. *glabrum* along with heptadecanoic acid (**2**). The structure of the new compound was elucidated by interpretation of its NMR (1D and 2D), MS, and IR data. Combined fractions *C* and *D* from the column chromatography of the hexane extract exhibited significant antifungal activities (average *MIC* of 0.10 mg/ml) against *Candida albicans* and *Cryptococcus neoformans. C. albicans* was relatively resistant to clerodendrumic acid (**1**; *MIC* 125 μg/ml) and was resistant to heptadecanoic acid (**2**; *MIC* 188 μg/ml). Both compounds had low antibacterial activities against two *Gram*-positive and two *Gram*-negative bacteria with average *MIC* values of 157 and 172 μg/ml, respectively. Compounds **1** and **2** were relatively nontoxic against monkey kidney *Vero* cells *in vitro* with IC_{50} values of 202.6 and 108.4 μg/ml, respectively.

Introduction. – The genus *Clerodendrum* in the Verbenaceae family which consists of *ca.* 580 species is widely distributed in Asia, Australia, America, and Africa [1]. *Clerodendrum glabrum* E.MEY. var. *glabrum*, known as white cat's whiskers or tinder wood and called '*Munukhatshilongwe*' in Venda, is a small to medium-sized deciduous tree, with the crown often drooping, which is widely distributed in Bushveld and along forest margins [2]. The leaves are used for treating roundworms and threadworm infections, as a wound dressing, as purgatives in domestic animals, for treatment of stomach troubles, coughs, and fevers, colds, mouth ulcers, sore throats, and intestinal worms in humans, while their roots are used for treatment of arthritis and fractured bones [3–5]. Their leaves have an unpleasant odor and are used as an insect repellent. Pounded leaves are placed in the armpit and neck to induce sleep and to provide a remedy for convulsions in children [6]. Previous phytochemical investigations on some species of the genus *Clerodendrum* led to the isolation of phenols, flavonoids, terpenoids, steroids, and cyanogenic glycosides [1][7][8].

Various local uses of the leaves of this species in addition to the search for bioactive chemical constituents from South African medicinal plants motivated our efforts to commence with a bioassay-guided fractionation of *C. glabrum* var. *glabrum*. To the best

of our knowledge, no phytochemical and biological work has been reported on the leaves of this species. Several of the traditional uses of this plant may be associated with antimicrobial activity. We, therefore, decided to isolate and characterize compounds with antifungal and antibacterial activity by bioassay-guided fractionation. To evaluate the potential use of an extract or compound, the cellular toxicity also has to be investigated. The therapeutic index, *i.e.*, ratio of activity against microorganisms to toxicity to animal cells, is important.

We report herein the isolation and characterization of one new triterpenoid, clerodendrumic acid (**1**), along with a known compound, **2**, from the leaves of *C. glabrum* var. *glabrum*, as well as the antimicrobial activities of fractions and constituents, and the cytotoxicity of the isolated compounds.

Results and Discussion. – *Characterization of Isolated Compounds.* The hexane crude extract of the leaves of *C. glabrum* was subjected to repeated silica-gel and *Sephadex LH-20* column chromatography to yield clerodendrumic acid (**1**) along with the known heptadecanoic acid (**2**). The antimicrobial and cytotoxicities of fractions, and isolated compounds **1** and **2** were determined.

Clerodendrumic acid (**1**) was obtained as a white powder and gave a positive result with the *Liebermann–Burchard* test characteristic for triterpenes. Its HR-ESI-MS exhibited a pseudo-molecular-ion ($[M+H]^+$) peak at *m/z* 573.4290 corresponding to the molecular formula $C_{34}H_{53}O_7$, in conjunction with the NMR data. The bands observed in its IR spectrum at 3300 and 1709 cm^{-1} are indicative of the presence of OH and C=O groups, respectively. The ^{1}H-NMR data (*Table 1*) of **1** showed four *singlet*s assignable to four Me groups (δ(H) 0.63, 1.00, 1.12, and 1.13), one Me *doublet* (δ(H) 1.96, $J = 6.0$), and signals of two oxygenated CH (δ(H) 4.51 (*dd*, $J = 7.1, 3.4$) and 4.56–4.58 (*m*)) and two exocyclic CH_2 groups (δ(H) 4.75 (br. *d*, $J = 1.3$) and 4.82 (br. *d*, $J = 1.2$)). The ^{13}C-NMR data (*Table 1*), in combination with DEPT and HSQC, revealed the presence of 34 C-atoms including nine quaternary C-atoms, and seven CH, twelve CH_2, and six Me groups. Signals due to two COOH and one ester C=O groups were observed at δ(C) 178.8 (C(28)), 178.7 (C(23)), and 169.5 (C(1′)), respectively, while signals due to oxygenated C-atoms were detected at δ(C) 79.1 (C(3)) and 77.7 (C(11); both CH C-atoms). The presence and location of the exocyclic CH_2 group in **1** was evidenced by the signals at δ(C) 103.2 (*t*, C(30)) and 154.5 (*s*, C(20)) in its ^{13}C-NMR spectrum, as well as the HMBCs between the H-atom signals at δ(H) 4.75 (H–C(30)) and 4.82 (H–C(30)), and C-atom signals at δ(C) 43.5 (C(19)) and 45.2 (C(21); *Fig. 1*).

Further important HMBCs between H-atom signals at δ(H) 1.12 (Me(24)) and 2.26–2.33 (H–C(5)), and C-atom signal at δ(C) 178.7 (C(23)), and between H-atom signals at δ(H) 1.74–1.81 (CH_2(22)) and 2.19–2.24 (H–C(18)) and C-atom signal at δ(C) 178.8 (C(28)) evidenced that the COOH groups were located at C(4) and C(17), respectively. HMBCs (*Fig. 1*) between the H-atom signal at δ(H) 4.51 (H–C(11)) and C-atom signals at δ(C) 43.9 (C(10)) and 48.6 (C(13)) indicated that the OH group was attached to C(11). Signals at δ(C) 38.0 (C(2′)), 32.2 (C(3′)), and 15.9 (C(4′)) were assignable to a propyl group whose presence was established from the HMBCs (*Fig. 1*) between H-atom signal at δ(H) 1.21–1.27 (H_a–C(2′)), and C-atom signals at δ(C) 169.5 (C(1′)) and 32.2 (C(3′)), and between H-atom signal at δ(H) 4.56–4.58 (H–C(3)) and C-atom signal at δ(C) 169.5 (C(1′)), which was linked to C(3).

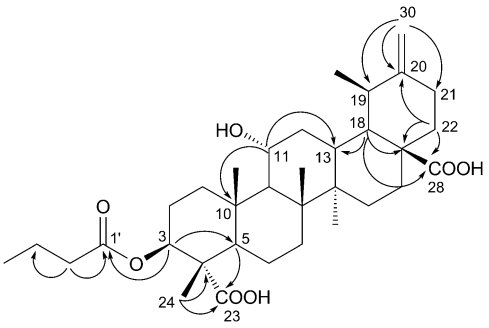

Fig. 1. *Key HMBCs of clerodendrumic acid* (**1**)

Table 1. *^{1}H- and ^{13}C-NMR* (600 and 150 MHz, resp., in $(CD_3)_2CO$) *Data for Clerodendrumic Acid* (**1**). δ in ppm, J in Hz.

Position	δ(H)	δ(C)
1	0.88–0.94 (*m*), 1.53–1.59 (*m*)	38.3
2	1.59–1.65 (*m*), 1.69–1.76 (*m*)	27.1
3	4.56–4.58 (*m*)	79.1
4		46.5
5	2.26–2.33 (*m*)	41.8
6		17.6
7	1.36–1.42 (*m*), 1.42–1.49 (*m*)	26.9
8		22.6
9	1.51–1.59 (*m*)	51.7
10		43.9
11	4.51 (*dd*, $J = 3.4, 7.1$)	77.7
12	1.26–1.31 (*m*), 1.52–1,58 (*m*)	45.0
13	1.58–1.64 (*m*)	48.6
14		46.1
15	0.89–0.97 (*m*), 1.86–1.90 (*m*)	39.4
16	1.47–1.51 (*m*), 1.69–1.77 (*m*)	33.2
17		46.9
18	2.19–2,24 (*m*)	42.4
19	2.06 (*d*, $J = 10.9$)	43.5
20		154.5
21	2.02–2.09 (*m*), 2.21–2.29 (*m*)	45.2
22	1.74–1.81 (*m*), 1.58–1.64 (*m*)	36.7
23		178.7
24	1.12 (*s*)	17.0
25	1.13 (*s*)	15.8
26	0.63 (*s*)	19.7
27	1.00 (*s*)	14.2
28		178.8
29	1.96 (*d*, $J = 6.0$)	20.2
30	4.75 (br. *d*, $J = 1.3$), 4.82 (br. *d*, $J = 1.2$)	103.2
1′		169.5
2′	1.21–1.27 (*m*), 1.92–1.99 (*m*)	38.0
3′	1.25–1.34 (*m*), 2.02–2.09 (*m*)	32.2
4′	1.09 (br. *s*)	15.9

Moreover, the presence of this group was supported by the ion-fragment peak $[M + H - CH_3CH_2CH_2COO]^+$ at *m/z* 486 (*Fig. 2*) in the ESI-MS spectrum. The α- and β-configurations of the OH group at C(11) and of the Me group at C(19), respectively, were confirmed by the ^3J(H,H) couplings ($J = 7.1, 3.4$ and $J = 10.9$) of the axial H-atoms H–C(11) and H–C(19) [9]. The ESI mass spectrum of **1** (*Fig. 2*) exhibited fragment-ion peaks in agreement with the proposed structure for clerodendrumic acid (**1**). From the above spectroscopic data and by comparison with those in the literature for pentacyclic taraxastane triterpenoids [9–11], the structure of clerodendrumic acid (**1**), isolated and characterized for the first time, was elucidated as (3β,11α,19β)-3-(butanoyloxy)-11-hydroxytaraxast-20(30)-ene-23,28-dioic acid.

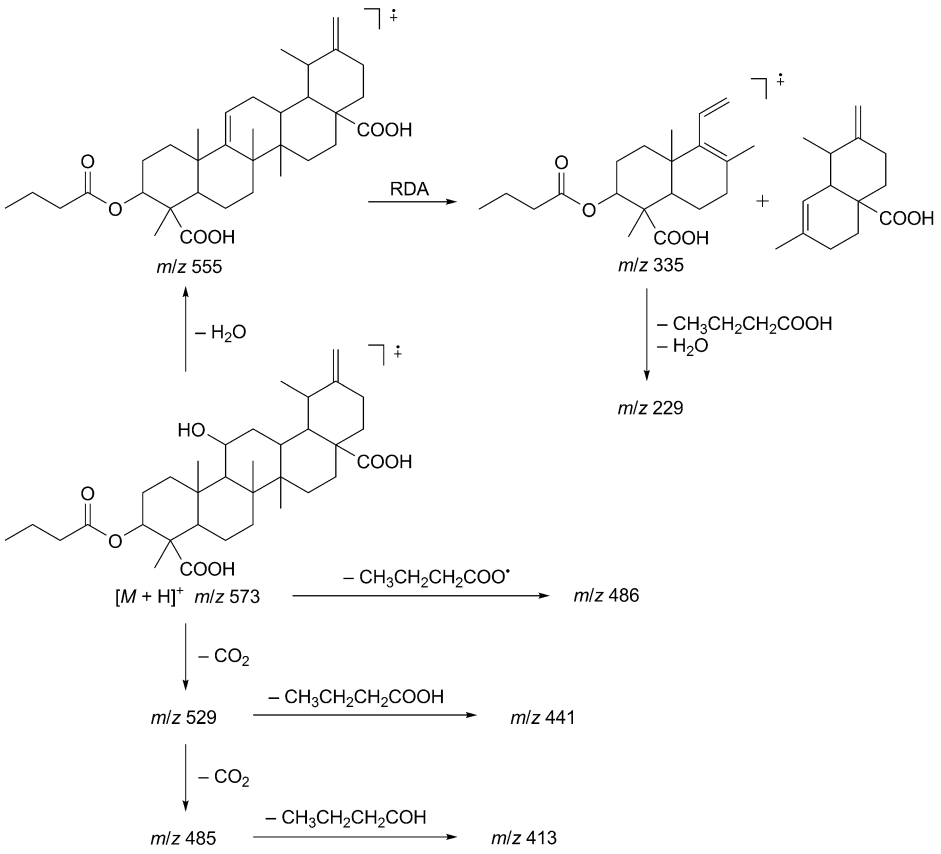

Fig. 2. *Significant ESI-MS fragmentations of clerodendrumic acid* (**1**)

Antimicrobial Activity. Minimum inhibition concentration (*MIC*) values of fractions and isolated compounds were determined against fungal and bacterial pathogens. Many authors consider antimicrobial activity of extracts to be significant if the *MIC* value is 0.1 mg/ml or lower [12][13]. Based on this criterion, *Frs. C* and *D* had significant activities against *C. albicans* and *C. neoformans* with average *MIC* values of 0.10 mg/ml (*Table 2*). This correlated with the results of bioautography showing clear

zones of inhibition against *C. albicans* (*Fig 3*). Total activity of extracts and fractions takes into account not only the *MIC* value of the extract or fraction, but also mass present in extract or fraction. The highest total activity was found in *Frs. C* and *E* with an average value of 15750 ml (*Table 3*). This value means that these fractions can be diluted to 15750 ml and will still kill the test organism [13].

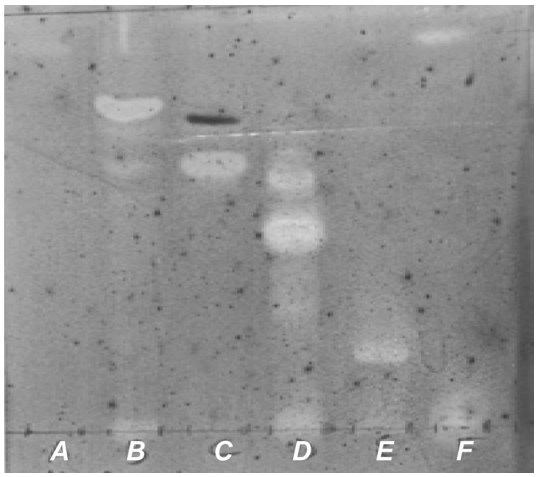

Fig. 3. *Anti*-Candida *activity of Fractions* A – F *from the extract of* C. glabrum *using bioautography technique on TLC plate sprayed with* Candida albicans *culture.* Heptadecanoic acid (**2**) was the main active compound in *Fr. C*, and clerodendrumic acid (**1**) was the main active compound in *Fr. D.*

According to *Rios* and *Recio* [14], the endpoint criteria for pure compounds isolated from plants with noteworthy activity is an *MIC* value of ≤ 10 μg/ml. *MIC* Values of the isolated compounds **1** and **2** against the test fungi and bacteria ranged from 125 to 188 μg/ml (*Table 2*), and clerodendrumic acid (**1**) had moderate activity against *C. albicans*, *C. neoformans*, and *A. niger* (*MIC* value of 125 μg/ml and total activity of 8 ml/mg). The three fungi were more resistant to compound **2** with an average *MIC* value of 167 μg/ml and a total activity of 6.2 ml/mg calculated as the inverse of the *MIC* (*Tables 2* and *3*). The positive control, amphotericin B, had a very low average *MIC* value of 0.65 μg/ml against the tested fungi, and this confirms its superiority over the isolated compounds as an antifungal agent.

The antibacterial activities of compounds **1** and **2** were weak against the tested microorganisms with *MIC* values of > 100 μg/ml [15]. The bacteria *P. aeruginosa* and *S. aureus* were more resistant to the compounds than the tested fungi with a *MIC* value of 188 μg/ml and total activity of 5.3 ml/mg (*Tables 2* and *3*). This trend of activity seems not to be related with the structure of the cell wall of the tested bacteria, as *P. aeruginosa* is *Gram*-negative while *S. aureus* is *Gram*-positive. This is in agreement with the results of other workers who reported that the compounds (terpenoids) inhibited the test fungi (*A. niger*, *C. albicans*, *etc.*) but not the bacteria (*S. aureus, S. epidermidis*, and *Bacillus subtilis*) [16]. However, it was also found that different compounds in the

Table 2. *Minimum Inhibitory Concentrations* (*MICs*) *of Fractions* A – F, *and Compounds* **1** *and* **2** *from* C. glabrum *against Fungi and Bacteria*. The results are the means of three replicates, and the standard deviation was zero.

Samples	Fungi[a])							Bacteria[a])								
	C.a.		*C.n.*		*A.n.*		Average	*S.a.*		*P.a.*		*E.f.*		*E.c.*		Average
	24 h	48 h	24 h	48 h	24 h	48 h		1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h	
Fractions [mg/ml]																
A	0.31	0.63	0.08	0.16	NT[b])	NT	0.30	0.63	0.63	0.63	1.25	0.63	0.63	1.25	1.25	0.86
B	0.08	0.16	0.08	0.16	NT	NT	0.12	0.63	0.63	1.25	1.25	0.63	1.25	1.25	1.25	1.02
C	0.08	0.16	0.08	0.08	NT	NT	0.10	0.31	0.31	0.31	0.31	0.16	0.16	0.31	0.31	0.27
D	0.08	0.16	0.08	0.08	NT	NT	0.10	0.16	0.31	0.31	0.31	0.08	0.16	0.31	0.63	0.28
E	0.08	0.16	0.08	0.16	NT	NT	0.12	0.16	0.16	0.31	0.63	0.31	0.31	0.31	0.63	0.35
F	0.31	0.63	0.63	0.08	NT	NT	0.55	0.63	0.63	0.63	1.25	0.63	1.25	1.25	1.25	0.94
Compounds [µg/ml]																
1	125	125	125	125	125	125	125	188	188	188	188	125	125	125	125	157
2	188	188	125	125	188	188	167	188	188	188	188	125	125	188	188	172
Amphotericin B[c])	0.02[d])	0.04[d])	0.04[d])	0.08[d])	NT	NT	0.05[d])	–	–	–	–	–	–	–	–	–
	0.78[e])	0.78[e])	0.78[e])	0.78[e])	0.39[e])	0.39[e])	0.65[e])									
Gentamicin[c])	–	–	–	–	–	–	–	0.39[d])	0.39[d])	0.39[d])	0.78[d])	0.78[d])	0.16[d])	0.78[d])	0.78[d])	0.56[d])
								0.19[e])	0.19[e])	0.78[e])	0.78[e])	0.39[e])	0.39[e])	0.59[e])	0.59[e])	0.49[e])

[a]) *C.a.*, *Candida albicans*; *C.n.*, *Cryptococcus neoformans*; *A.n.*, *Aspergillus niger*; *S.a.*, *Staphylococcus aureus*; *P.a.*, *Pseudomonas aeruginosa*; *E.f.*, *Enterococcus faecalis*; *E.c.*, *Escherichia coli*. [b]) NT, not tested. [c]) Positive control. [d]) *MIC* Values for reference standard when testing compounds. [e]) *MIC* Values for reference standard when testing fractions.

Table 3. *Total Activity* [ml] *of Fractions* A – F *from* C. glabrum *Calculated by Dividing Mass of Fraction with the* MIC *Value of the Fraction* [13], *and 1/*MIC [ml/mg] *Calculated for Compounds* **1** *and* **2**. This indicated to what volume 1 mg of the compound can be diluted and still kills the relevant microorganism.

Samples	Fungi[a])							Bacteria[a])								
	C.a.		*C.n.*		*A.n.*		Average	*S.a.*		*P.a.*		*E.f.*		*E.c.*		Average
	24 h	48 h	24 h	48 h	24 h	48 h		1 h	2 h	1 h	2 h	1 h	2 h	1 h	2 h	
Fractions (Total activity [ml])																
A	4839	2381	18750	9375	NT[b])	NT[b])	8836	7730	7730	7730	3896	7730	7730	3896	3896	6292
B	15000	7500	15000	7500	NT[b])	NT[b])	11250	1905	1905	960	960	1905	960	960	960	1314
C	18000	9000	18000	18000	NT[b])	NT[b])	15750	4645	4645	4645	4645	9000	9000	4645	4645	5734
D	17750	8875	17750	8875	NT[b])	NT[b])	13313	8875	4581	4581	4581	17750	4581	4581	2254	6473
E	18000	9000	18000	18000	NT[b])	NT[b])	15750	9000	9000	4645	2285	4645	4645	4645	2285	4858
F	290	143	143	143	NT[b])	NT[b])	180	290	143	143	72	290	143	72	72	153
Compounds (1/*MIC* [ml/mg])																
1	8	8	8	8	8	8	8	5.3	5.3	5.3	5.3	8	8	8	8	6.7
2	5.3	5.3	8	8	5.3	5.3	6.2	5.3	5.3	5.3	5.3	8	8	5.3	5.3	6

[a]) *C.a.*, *Candida albicans*; *C.n.*, *Cryptococcus neoformans*; *A.n.*, *Aspergillus niger*; *S.a.*, *Staphylococcus aureus*; *P.a.*, *Pseudomonas aeruginosa*; *E.f.*, *Enterococcus faecalis*; *E.c.*, *Escherichia coli*. [b]) NT, not tested.

tested fractions were active against bacterial species (*S. aureus, E. coli, P. aeruginosa*, and *E. faecalis*) in bioautography (*Fig. 3*).

The antifungal activities of *Frs. C* and *D* from which the compounds were isolated, were higher than those of the isolated compounds (*Table 2*). This suggests the presence of synergistic interactions of different compounds in the respective fractions. Weak potency of the compounds may also be due to some decomposition which might have occurred during the isolation process [17]. In this case, the isolated compounds had the same R_f value as the active compounds in the fractions which makes it unlikely that an artifact has been isolated (*Fig. 3*). However, further investigation could be conducted especially on the novel compound, clerodendrumic acid (**1**), to potentiate its biological activity. For instance, it can either be used together with another compound in order to increase its activity, or, due to its likely safety, it can be used together with known antibiotics to boost their potency and to mitigate their undesirable side effects [14].

The isolated compounds did not show appreciable activity against the tested pathogens. Our group has found that, in some cases, crude extract or fractions have an excellent potential for treating microbial infections, and may even have as good or higher activity than commercially used antimicrobials in field [18] or clinical [19] trials. Major or minor constituents in the plant extract interacting in a synergistic manner may not be highly active, when they are not part of a mixture with synergistic compounds. To the best of our knowledge, the biological activities of the fractions and isolated compounds from the leaves of this plant against the selected bacteria and fungi are being reported for the first time.

Cytotoxicity of Isolated Compounds. Not only the antimicrobial activity is important, but also the cellular toxicity. If a compound has rather low activity but much lower toxicity to animal cells, it may still be useful. Compounds **1** and **2** were evaluated for their cytotoxic activities against monkey kidney *Vero* cells *in vitro* by means of the MTT (= 3-(4,5-dimethylythiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay and were found relatively inactive in inhibiting the proliferation of *Vero* cells with IC_{50} values >100 μg/ml (*Table 4*). According to the *American National Cancer Institute* (*NCI*), the criterion of cytotoxic activity for pure compounds is $IC_{50} \leq 4$ μg/ml [20]. Thus, compounds **1** and **2** were relatively nontoxic, because they had IC_{50} values of 202.6 and 108.4 μg/ml, respectively, compared to the positive control doxorubicin (IC_{50} value of 9.9 μg/ml). The higher IC_{50} values of compounds **1** and **2** indicate their probable safety in terms of cytotoxicity, but more cell lines should be included in the comprehensive screening to confirm these results. Selectivity-index (*SI*) values, ranging from 0.6 to 1.6, of these compounds were poor. It is generally considered that the ratio for a good therapeutic index for a remedy or drug should be ≥ 10, which is a cut-off point ensuring that overdose does not put the life of the patient in danger [21]. To the best of our knowledge, there are no other reports on the cytotoxicity of these isolated compounds against *Vero* cells.

Conclusions. – The novel compound clerodendrumic acid (**1**) and a known compound, heptadecanoic acid (**2**), were isolated for the first time from the leaves of *C. glabrum. C. albicans* was relatively resistant to clerodendrumic acid (**1**) with a *MIC* value of 125 μg/ml and was resistant to heptadecanoic acid (**2**) with a *MIC* value of 188 μg/ml. The low antifungal activity of **1** disqualifies it as a feasible antifungal

Table 4. *Cytotoxicity of Compounds* **1** *and* **2** *from* C. glabrum *and Their Selectivity Indices against Three Fungal and Four Bacterial Species*

Compounds	Cytotoxicity		Selectivity index[a])						
	IC_{50} [μg/ml]	SD	*C.a.*	*C.n.*	*A.n.*	*S.a.*	*P.a.*	*E.f.*	*E.c.*
1	202.6	±3.6	1.6	1.6	1.6	1.1	1.1	1.6	1.6
2	108.4	±3.7	0.9	0.9	0.9	0.6	0.6	1.6	0.6
Doxorubicin[b])	9.9	±0.1							

[a]) *C.a.*, *Candida albicans*; *C.n.*, *Cryptococcus neoformans*; *A.n.*, *Aspergillus niger*; *S.a.*, *Staphylococcus aureus*; *P.a.*, *Pseudomonas aeruginosa*; *E.f.*, *Enterococcus faecalis*; *E.c.*, *Escherichia coli*. [b]) Positive control.

compound. However, due to its relatively low toxicity to animal cells, **1** could either be used as a lead compound to produce new chemically modified active derivatives to fight microbial infections, or could be used together with known antibiotics to mitigate their undesirable side effects. Other active compounds visualized in bioautography remain to be isolated and identified, and these may possibly contribute more to the activity of the crude extract and fractions than the compounds isolated in this study.

Experimental Part

General. Column chromatography (CC: *MN* silica gel *60* (0.063–0.2 mm/70–230 mesh). TLC: Precoated silica gel *60* F_{254} (*Merck*, Germany) plates; visualization with UV light (254 and 365 nm), then spraying with vanillin/sulfuric acid spray reagent, followed by heating to 110° for 3 min. IR Spectra: *Bruker Alpha FT-IR* spectrometer (*Optik GmbH*, Germany); $\tilde{\nu}$ in cm^{-1}. ^{1}H- and ^{13}C-NMR spectra: *Varian* spectrometer at 600 MHz; chemical shifts (δ) in ppm with Me_4Si as internal standard; *J* in Hz. ESI-MS: *Waters Synapt HDMS* spectrometer; in *m/z* (rel. int.).

Plant Material. The leaves of *Clerodendrum glabrum* var. *glabrum* were collected in October 2009 at Tshipise (Limpopo province of South Africa). The plant was identified by Mr. *M. P. Tshisikhawe*, botanist at the University of Venda (South Africa), where the specimen was deposited with the herbarium of the Department of Botany under the voucher No. Rn 46.

Extraction and Isolation. The dried and powdered leaves of *C. glabrum* (2.85 kg) were extracted with 5 l of hexane for 5 h under shaking and at r.t. to afford a crude extract (34.59 g) after filtration and concentration *in vacuo*. A part of the crude extract (10 g) was subjected to CC (silica gel (45 cm × 100 mm, 938 g); hexane/AcOEt in increasing polarity (0 to 100%)) to give 84 fractions of 300 ml each that were combined using comparative TLC into six main fractions: *A* (*1–14*; 1.5 g), *B* (*15–17*; 1.2 g), *C* (*18–31*; 1.44 g), *D* (*32–38*; 1.78 g), *E* (*39–63*; 1.44 g), *F* (*64–84*; 0.09 g). *Frs. A*, *B*, *E*, and *F* did not have as many and as active antimicrobial compounds separated by TLC in bioautography as *Frs. C* and *D*, and were not further investigated.

Fr. D (1.78 g) was subjected to CC (silica gel (50 cm × 2 cm, 70 g); hexane/AcOEt in increasing polarity) to give 87 fractions of 50 ml each that were monitored with co-TLC to nine subfractions. *Subfr.* D_4 (0.34 g) was subjected to further CC (hexane/AcOEt from 96 : 4 to 80 : 20) to afford 41 fractions of 30 ml each. *Subfrs.* $D4_{13–18}$ from D_4 gave mainly **1** (19.4 mg). *Fr. C* (1.44 g) was similarly subjected to CC as described for *Fr. D* to afford **2** (24.7 mg).

Clerodendrumic Acid (*=(3β,11α,19β)-3-(Butanoyloxy)-11-hydroxytaraxast-20(30)-ene-23,28-dioic Acid=(3β,11α,18α)-3-(Butanoyloxy)-11-hydroxyurs-20(30)-ene-23,28-dioic Acid*; **1**). White powder. $[\alpha]_{546}^{20} = -10.6$ (*c* = 1.7, $(CD_3)_2CO$). IR: 3300, 2900, 1709, 1442, 982. ^{1}H- (600 MHz) and ^{13}C-NMR (150 MHz) in (D_6)acetone: *Table 1*. ESI-MS: 573 ($[M+H]^+$, 42), 555 (8), 529 (40), 486 (13), 485 (25),

441 (16), 413 (35), 368 (37), 273 (26), 229 (100). HR-ESI-MS: 573.4290 ($[M+H]^+$, $C_{34}H_{53}O_7^+$; calc. 573.4308).

Antimicrobial Assay. The twofold serial microdilution method was used to determine the minimum inhibitory concentration (*MIC*) values for fractions and isolated compounds against bacteria [22], and for fungi a modification of the procedure in [22] by *Masoko et al.* was applied [23]. The *MIC* values of the samples were evaluated using two *Gram*-positive bacteria, *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), two *Gram*-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922); and the pathogenic fungi *Candida albicans*, *Cryptococcus neoformans* (clinical isolates), and *Aspergillus niger*.

In the antibacterial tests, 100 µl of fractions (10 mg/ml) or compounds (4 mg/ml) in triplicate dissolved in acetone were serially diluted twofold with sterile dist. H_2O in 96-well microtitre plates, and 100 µl of freshly prepared overnight bacterial culture in *Mueller–Hinton* broth (*Fluka*, Switzerland) were added to each well. Approximate densities of bacterial cultures used were: 2.6×10^{12} cfu/ml for *S. aureus*, 1.5×10^{10} cfu/ml for *E. faecalis*, 5.2×10^{13} cfu/ml for *P. aeruginosa*, and 3.0×10^{11} cfu/ml for *E. coli* [24]. Acetone and gentamicin were used as negative and positive controls, resp. The microtitre plates were sealed in air-tight polythene plastic bags and were incubated overnight at 37°. Thereafter, 40 µl of 0.2 mg/ml of *p*-iodonitrotetrazolium violet (INT) were added to each well to indicate microbial growth. The microtitre plates were further incubated at 37°, and *MIC* values were determined 1 and 2 h after the addition of INT. The *MIC* values was determined as the lowest concentration inhibiting microbial growth, indicated by a decrease in the intensity of the red color of the formazan [25].

For the antifungal assay, fractions and isolated compounds were dissolved in acetone to a concentration of 10 and 4 mg/ml, resp. Fungal cultures were taken from agar culture plates and inoculated in fresh *Sabouraud* dextrose broth and incubated at 37° for 3 d prior to conducting the assay. Approximate densities of fungal cultures used were: 2.5×10^4 cfu/ml for *C. albicans*, 2.6×10^4 cfu/ml for *C. neoformans*, and 1×10^6 cfu/ml for *Aspergillus niger* [24]. The fractions or compounds (100 µl) in triplicate were serially diluted twofold with sterile dist. H_2O in 96-well microtitre plates. Thereafter, 100 µl of the fungal culture were added to each well. Amphotericin B and acetone were used as positive and negative controls, resp. To indicate growth of microorganisms, 40 µl of 0.2 mg/ml of INT were added to each well. The microplates were put in air-tight plastic bags and then incubated for 2 d at 37° in 100% relative humidity. *MIC* was taken as the lowest concentration of the extract that inhibited fungal growth after 24 and 48 h. The experiment was conducted twice in order to verify the results.

Cytotoxicity Assay. The MTT (= 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-2*H*-tetrazolium bromide) reduction assay is widely used for measuring cell proliferation and cytotoxicity. MTT (yellow) is reduced into a formazan (purple) by viable cells. The color intensity of the formazan produced, which is directly proportional to the number of viable cells, is measured using a spectrophotometer. Viable-cell growth after incubation with known concentration of either fractions of plant extract or isolated compound was determined using the colorimetric MTT assay as described by *Mosmann* [26]. Monkey *Vero* cells of a subconfluent culture were harvested and centrifuged at 200 *g* for 5 min, and resuspended in growth medium to a density of 0.1×10^6 cells/ml. Minimal Essential Medium (MEM, *Sigma*) supplemented with 0.1% gentamicin and 5% foetal calf serum (*Sigma*) was used. A total of 100 µl of cell suspension was added into each well of columns *2* to *11* of a sterile 96-well microtitre plates. Growth medium (200 µl) was added into wells of columns *1* and *12* to minimize the 'edge effect' and maintain humidity. The plates were incubated for 24 h at 37° in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. Test samples or doxorubicin (*Sigma*; as the positive control prepared in growth medium (100 µl)) at various known concentrations were added to each well (in quadruplicate, except for column *2* where only growth medium was added). The microtitre plates containing treated and untreated cells were incubated at 37° in a 5% CO_2 incubator for 2 d with the various treatments. After incubation, the medium was removed from each well, and each well was rinsed with PBS (= phospate-buffered saline) before fresh growth medium (200 µl) was added. Following this washing step, 30 µl MTT (*Sigma*; stock soln. of 5 mg/ml in PBS) were added to each well, and the plates were incubated for a further 4 h at 37°. The medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well, followed by gentle shaking of the MTT soln. The amount of MTT reduction was measured immediately by detecting absorbance at 570 nm

using a microplate reader (*Versamax*). The wells in column *1*, containing only medium and MTT but no cells, were used to blank the reader. The IC_{50} values were calculated as the concentration of plant extract or test compound resulting in a 50% reduction of absorbance compared to untreated cells. Selectivity index (*SI*) of the isolated compounds was calculated as follows: $SI = IC_{50}/MIC$.

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