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Antimosquito and Antimicrobial Clerodanoids and a Chlorobenzenoid from *Tessmannia* species

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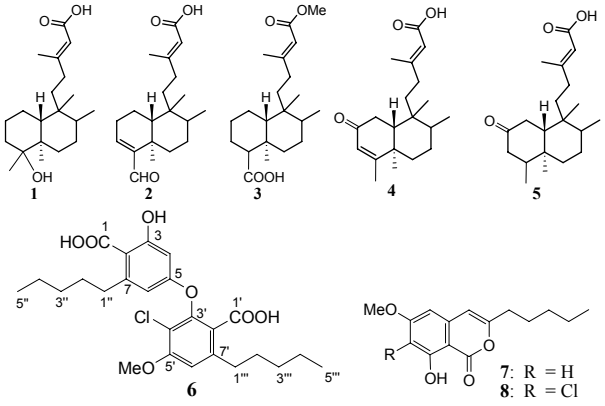
The clerodane diterpenoids *trans*-kolavenolic acid, 18-oxocleroda-3,13(*E*)-dien-15-oic acid, *ent*-(18-hydroxycarbonyl)-cleroda-3,13(*E*)-dien-15-oate, 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid and *trans*-2-oxo-*ent*-cleroda-13(*Z*)-en-15-oic acid, and the chlorobenzenoid *O*-(3-hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-6-pentyl-2-oxybenzoic acid were isolated from *Tessmannia martiniana* var *pauloi* and *T. martiniana* var *matiniana.* Structures were established based on interpretation of spectroscopic data. Some of the compounds exhibited significant antimosquito, antifungal and antibacterial activities.

Keywords: *Tessmannia martiniana* var *pauloi*, *T. martiniana* var *martiniana*, Caesalpiniaceae, *ent*-clerodanoids, chlorobenzenoid, anti-mosquitoes, antimicrobials.

In our previous paper [1] we reported the isolation, structural determination, insect repellency and antimicrobial activity of *nor*-halimanoid diterpenes and some other compounds from *Tessmannia densiflora*. In continuation with investigations of Tanzania *Tessmannia* species for their antimosquito, antimicrobial and other constituents we now analyzed the root and stem barks of *T. martiniana* var *pauloi* Harms and *T. martiniana* var *martiniana* Harms. In Tanzania the plant species grow in the coastal evergreen forest reserves of Pugu and Zaraninge, respectively. None of them has previously been investigated chemically for bioactive or any other constituents. We now report the isolation and antimosquito, antibacterial and antifungal activities of *ent*-clerodane diterpenoids and a chlorobenzenoid from these plant species.

The larvicidal methanol extracts from the root bark of *T. martiniana* var *pauloi* on repeated chromatography yielded *trans*-kolavenolic acid (1) [2], 18-oxocleroda-3,13(*E*)-dien-15-oic acid (2), which was previously



isolated as an antifeedant constituent of *Detarium microcarpum* [3], and *ent*-(18-hydroxy-carbonyl)-cleroda-3,13(*E*)-dien-15-oate (3) [4]. Similarly, an active chloroform extract of the root bark of *T. martiniana* var *martiniana*, apart from 3, also gave 2-oxo-*ent*-cleroda-3,13(*Z*)-dien-15-oic acid (4) [5], and 2-oxo-*ent*-cleroda-13(*Z*)-dien-15-oic acid (5), while the

Table 1: ^{1}H and ^{13}C NMR spectral data for the chlorobenzenoid **6**.

H/C	δ_H	*J* (Hz)	δ_C	COSY	HMBC
1	-		174.7		
2	-		108.6		
3	-		165.2		
4	6.75	d, 2.1	108.6	H-6	C-2, C-3, C-5, C-6
5	-		154.7		
6	6.63	d, 2.4	115.9	H-4	C-2, C-4, C-5, C-1"
7	-		146.8		
1'	-		169.2		
2'	-		105.2		
3'	-		160.5		
4'	-		107.9		
5'	-		159.8		
6'	6.44	s	106.5		C-2', C-4', C-5', C-1"'
7'	-		150.1		
1"	3.01	m	36.5	H-2"	C-2, C-6, C-7, C-2", C-3"
2"	1.68	m	32.0	H-1", H-3"	C-1", C-4"
3"	1.34	m	31.3	H-2", H-4"	C-2", C-4"
4"	1.34	m	22.5	H-3", H-5"	C-2", C-4", C-5"
5"	0.91	t, 7.5	14.0	H-4"	C-2", C-3", C-4"
1"'	3.01	m	37.6	H-2"'	C-2', C-6', C-7', C-2"', C-3"
2"'	1.68	m	32.1	H-1"', H-3"'	C-1"', C-4"'
3"'	1.34	m	31.9	H-2"', H-4"'	C-2"', C-4"'
4"'	1.34	m	22.4	H-3"', H-5"'	C-2"', C-4"', C-5"'
5"'	0.88	t, 7.0	14.0	H-4"'	C-2"', C-3"', C-4"'
OMe	3.99	s	56.3		C-5'
COOH	11.30	br s			
OH	11.70	br s			

new chlorobenzenoid (6) was obtained from stem bark of *T. martiniana var martiniana*.

Structure **6** for the new chlorobenzenoid was established on the basis of its ^{1}H and ^{13}C NMR spectral data (Table 1) and MS, all of which indicated that the compound consisted of two units derived from **7** and **8** that we recently obtained from *T. densiflora* [1]. The presence of a chlorine atom was deduced from the high resolution EIMS that showed peaks at *m/z* 477.1626 and 479.1635 corresponding to the $[M–1]^+$ and $[M+1]^+$ fragment ions, and 3:1 relative intensity ratio, which corresponds to the natural abundance of Cl isotopes {calculated for $C_{25}H_{30}ClO_7$ = 477.1680 corresponding to $[M-1]^+$}. The position of Cl was derived from the MS fragmentation pattern and upon analysis of HMBC interactions (Table 1), which also indicated the relative positions of all the protonated C substituents and hence confirming structure **6** for the isolated compound.

When assayed for mosquito larvicidal properties the *ent*-clerodanoids **2**–**5** and a chlorobenzenoid **6** showed moderate activity after 24 h larvae exposure, their efficacy being enhanced with prolonged time of exposure to 48 and 72 h (Table 2). However, the compounds exhibited insignificant difference in activity compared with the crude extract after 24 h [6].

Compounds **2** and **3** (Table 2), as well as their crude extract, exhibited stronger activity than their refined precursor fractions. This suggested that the efficacy of the active principles **2** and **3** could have been masked by impurities in the semi-purified fractions. On the other hand, the stronger activity of the crude extract was presumed to result from synergistic effects not only involving **2** and **3**, but also other compounds such as **1** and others, which occurred in small amounts and hence could not be isolated. The enhanced activity of the crude extract could also be attributed to decomposition products formed during the isolation process, as has been previously observed elsewhere [7]. However, the fact that the crude extract displayed very interesting insect growth regulatory and larvicidal effects [6] indicates that whether in crude extract, refined fractions or as the pure compounds, the constituents of *T. martiniana* var *pauloi* root barks are potential botanical mosquito larvicides.

Compound **3**, which was obtained in appreciable amounts, was also assayed for mosquito repellency, for which it exhibited very low activity, being less than 50% as active as DEET.

Table 2 shows that of compounds **2** – **6** assayed for larvicidal activity, compound **3** was the most active, being nearly three times more active than its crude extract after 24 h larvae exposure (methanol extract of the root bark of *T. martiniana* var *pauloi* and chloroform extract of the stem bark *T. martiniana* var *martiniana*). Compound **5** was the least active, being four times less potent than its crude extract after 24 h larvae exposure. The higher larvicidal efficacy of compounds **2** – **4** compared with **5** could be attributed to the presence of an α,β-unsaturated carbonyl system in **2** – **4**, whose enhanced contribution to bioactivity has previously been reported [7,8].

The cleradonoids **2** and **3** also showed antimicrobial activity at different levels against both Gram-positive and Gram-negative bacterial strains, as well as against the tested fungal species. Compound **2** was the least active; it exhibited activity only against the Gram-positive bacterium *B. subtilis* and the filamentous fungus *Aspergillus niger* at a level lower than that shown by the standard antibiotic and antifungal agent Ampicillin and Fluconazole, respectively. Compound **3** showed activity against the three bacterial species *P. aeruginosa*, *S. aureus* and *B. subtilis* and for the l atter, the activity being comparable to that of the standard antibiotic Ampicillin. Compounds **1**, **4** – **6** could not be tested for antimicrobial activity due to paucity of the isolated amounts. These results further demonstrate the versatility of the family Caesalpiniaceae in accumulating bioactive metabolites of interest to biomedical research.

Experimental

General experimental procedures: CC: silica gel 60 (0.063-0.200 mm, Merck); TLC: silica gel 60 F_{254} (Merck) precoated plastic plates; visualization: UV-Vis and anisaldehyde spray [9]; IR: $CHCl_3$ or KBr; specific

Table 2: Activity (% mortality) of **2** – **6** and crude extracts against *Anopheles gambiae* larvae.

Cp	T (h)	Concentration (ppm)						LC_{50} (ppm) 95% CL
		15.62	31.25	62.5	125	250	500	
2	24	nd	nd	33.3 ± 3.3	46.7 ± 3.3	80 ± 5.7	90 ± 5.7	125 (57-204)
	48	nd	nd	53.3 ± 8.8	63.3 ± 3.3	96.7 ± 3.3	96.7 ± 3.3	~ 57 (nd)
	72	nd	nd	70 ± 5.7	90 ± 5.7	100 ± 0	100 ± 0	~ 11 (nd)
3	24	16.7±3.3	30 ±5.7	50 ± 0	83.3±3.3	100 ± 0	100 ± 0	48 (29-73)
	48	46.7±3.3	70 ±5.7	90 ± 5.7	100 ± 0	100 ± 0	100 ± 0	19 (7-29)
	72	83.3±3.3	86.7±3.3	100 ± 0	100 ± 0	100 ± 0	100 ± 0	~ 1 (nd)
4	24	nd	nd	20 ± 5.7	33.3±3.3	46.7 ±3.3	80 ± 5.7	212 (114-520)
	48	nd	nd	50 ± 5.7	70 ± 0	73.3 ±3.3	100 ± 0	~ 55 (nd)
	72	nd	nd	80 ± 5.7	100 ± 0	100 ± 0	100 ± 0	~ 22 (nd)
5	24	nd	nd	10 ± 1	16.7 ±6.7	20 ± 5.7	36.7 ±3.3	~ 737 (nd)
	48	nd	nd	16.7±8.8	33.3±8.8	36.7 ± 12	66.7 ±3.3	~ 345 (nd)
	72	nd	nd	20 ± 5.7	43.3 ±12	36.7 ± 12	80 ± 5.7	256 (149-708)
6	24	16.7±3.3	50 ± 0	53.3±3.3	60 ± 5.7	70 ± 5.7	100 ± 0	62 (30-111)
	48	50 ± 5.7	83.3 ±3.3	100 ± 0	80 ± 5.7	100 ± 0	100 ± 0	15 (2-26)
	72	60 ± 5.7	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	~ 3 (nd)
TMRM*	24	-	-	-	-	-	-	114 (44-186)
TMMRC*	24	-	-	-	-	-	-	204 (133-340)
TMMSC*	24	-	-	-	-	-	-	256 (149-708)

Cp = compound; CL = Confidence limits; nd = not determined; TMRM = *T. martiniana* var *pauloi* root bark crude methanol extract; TMMRC = *T. martiniana* var *martiniana* root bark crude chloroform extract; TMMSC = *T. martiniana* var *martiniana* stem bark crude chloroform extract; * quoted from ref. [6].

rotation: $CHCl_3$; 1D NMR: 300 or 500 MHz (^{1}H), and 75 or 125 MHz (^{13}C); 2D NMR (HMQC, HMBC, COSY, NOESY) at 500 MHz (^{1}H); chemical shift in ppm referenced to internal standard TMS (δ = 0) for ^{1}H and $CDCl_3$ (δ = 77.0 ppm) for ^{13}C NMR; MS: DSQII (Axel Semrau GmbH), GC-TOF (micromass) and Q-TOF micro (micromass) equipment.

Plant materials: The root and stem barks of *Tessmannia martiniana* var *pauloi* and *T. martiniana* var *martiniana* were collected in March 2006 from Pugu and Zaraninge Forest Reserves, respectively in Kisarawe and Bagamoyo Districts in Tanzania. The plant species were authenticated at the Herbarium of the Department of Botany at the University of Dar es Salaam, Tanzania, where voucher specimens are preserved under reference numbers FMM 1321 and 3374 respectively.

Extraction and isolation: The air-dried and pulverized *T. martiniana* var *martiniana* root and stem barks were (1.0 and 1.5 Kg respectively) extracted sequentially with $CHCl_3$ and MeOH (2 x 48 h for each solvent). The air-dried and pulverized *T. martiniana* var *pauloi* root bark (560 g) was extracted only with MeOH (2 x 48 h) due to paucity of the available plant materials. All the extracts were kept at -20°C until the isolation process was undertaken. The *T. martiniana* var *pauloi* MeOH extract (20 g), *T. martiniana* var *martiniana* root bark $CHCl_3$ extract (25 g) and the *T. martiniana* var *martiniana* stem bark $CHCl_3$ extract (25 g) that showed larvicidal activity, on bioassay guided fractionation by vacuum liquid chromatography (VLC), and then repeated column chromatography on silica gel (light pet./EtOAc gradient elution), and Sephadex® LH-20 (MeOH/$CHCl_3$, 1:1 v/v) yielded compounds **1**, **2** and **3** (from *T. martiniana* var *pauloi* MeOH extract); **3**, **4** and **5** (from *T. martiniana* var *martiniana* root bark chloroform extract) and **6** from *T. martiniana* var *martiniana* stem bark chloroform extract, while constituents of several other active fractions decomposed during the isolation process.

***O*-(3-Hydroxy-4-hydroxycarbonyl-5-pentylphenyl)-3-chloro-4-methoxy-6-pentyl-2-oxybenzoic acid** (**6**)

MP: 149°C.
Yield: 264 mg (0.018%).
Anisaldehyde: red.
^{1}H and ^{13}C NMR: Table 1.
MS, m/z (% rel. int.) 479 ($[M+1]^+$, 15), 477 ($[M-1]^+$, 45), 223 (55), 205 (100) and 179 (25).
HRMS, *m/z* 477.1626 and 479.1635 ($[M-1]^+$ and $[M+1]^+$, 3:1 ratio corresponding to the natural abundance of Cl isotopes).

Biological assay: Larvicidal, antibacterial and antifungal assays were carried out as reported in the literature [10-12]. In the larvicidal assays 20 late 3rd or young 4th instar larvae of *Anopheles gambiae* s.s were used per beaker with 3 beakers per concentration (the water temperature being 25 ± 1°C) and for each test 3 beakers containing distilled water and test larvae, but without sample, were used as controls. Observation on mortality and deformities of the larvae was recorded after every 24 h of continuous exposure and expressed as percent mortality [11]. The lethal concentration at which 50% of the test larvae were killed (LC_{50}) was determined using POLO PLUS computer package. The disc diffusion method was used in the antibacterial assay. *Staphylococcus aureus* and *Bacillus subtilis* were

used as the Gram-positive bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococus faecalis, Klebsiella pneumoniea* and *Salmonella typhimurium* as the Gram-negative bacteria strains, and Ampicillin (10 μg/mL) and Gentamycin (10 μg/mL) were used as the standard antibiotics. *Aspergillus fumigatus*, *A. niger* and *Candida albicans* were used in the antifungal tests and Fluconazole (20 μg/mL) as the standard antifungal agent. The assays were conducted in triplicate at 10 mg/mL concentration of each tested compound.

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