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Antifungal rosane diterpenes and other constituents of *Hugonia castaneifolia*

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Abstract

The rosane diterpenoids hugorosenone [3β-hydroxyrosa-1(10),15-dien-2-one], 18-hydroxyhugorosenone and 18-hydroxy-3-deoxyhugorosenone, and 12-hydroxy-13-methylpodocarpa-8,11,13-trien-3-one were isolated as antifungal constituents of *H. castaneifolia* Engl. root bark, together with the previously reported di-podocarpanoids hugonone A and hugonone B that were weakly active, and 1(10),15-rosadiene-2β,3β-diol (hugorosenol), 4α-methoxyhimachal-10-en-5β-ol (hugonianene B) and 2-hydroxyhenpentacont-2-enal, and the known compounds tetracosyl-(*E*)-ferrulate and caryophyllene oxide, all of which were inactive. Hugorosenone also exhibited activity against *Anopheles gambiae* mosquito larvae. Structural determination was achieved based on spectroscopic data.

Keywords: *Hugonia castaneifolia*; Linaceae; Antifungal; Larvicidal; Rosane diterpenoids; Himachalenoid; 2-Hydroxyhenpentacont-2-enal

1. Introduction

The Linaceae is a relatively small plant family that consists of only three genera, among which is the genus *Hugonia* 30 of whose 34 species are found in the tropical regions of continental Africa, Madagascar and Mauritius (Doreen, 1966; Friedman, 1987; Hutchings, 1996). Some of these species, including *H. castaneifolia* Engl., are used as herbal remedies (Kokwaro, 1976; Hutchings, 1996). This, and the fact that previously some *Hugonia* species yielded lignans related to the antitumour agent podophyllotoxin (Konuklugi, 1996) prompted us a few years ago to investigate the root bark of *H. castaneifolia* that occurs in East Africa, for cytotoxic and other constituents. In 1998, we reported the isolation of the cytotoxic rosane diterpenoid hugorosenone (3β-hydroxyrosa-1(10),15-dien-2-one, **1**), together with 18-hydroxyhugorosenone (**2**) and hugorosediol [1(10),15-rosadiene-3β,18-diol, and two di-podocarpanoids that were conceived to be products of Diels–Alder type cycloaddition reactions, originating from enzymatic or non-enzymatic dimerisation of the corresponding podocarpane monomers as both dienes and dienophiles (Mdee et al., 1998). As part of our on-going search for antifungal and mosquito larvicidal compounds from Tanzanian plants, we recently investigated active extracts from *H. castaneifolia*. We now hereby report results from these investigations.

2. Results and discussion

Repeated chromatography of the *n*-hexane and dichloromethane root bark extracts that showed activity against the fungus *Cladosporium cucumericum* Ell. Et Arth. and *Anopheles gambiae* s.s. mosquito larvae, gave compounds **1**, **2** and 18-hydroxy-3-deoxyhugorosenone (**3**) as anti-

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fungal constituents, and the inactive metabolites 1(10), 15-rosadiene-2β,3β-diol (hugorosenol, **4**), 4α-methoxyhimachal-10-en-5β-ol (hugonianene B, **5**), 2-hydroxyhenpentacont-2-enal (**6**), caryophyllene oxide (Connolly and Hill, 1991; Reina et al., 2002) and tetracosyl-(*E*)-ferrulate (Mensah et al., 1992), in addition to another active compound 12-hydroxy-13-methylpodocarpa-8,11,13-trien-3-one (**7**) (Itokawa et al., 1991) and the weakly active di-podocarpanoids hugonone A (**8**) and B (**9**) (Mdee et al., 1998).

The spectral properties of the diterpenoids **1**, **2**, **7**–**9**, caryophyllene oxide, and tetracosyl-(*E*)-ferrulate were comparable with those reported for these compounds (Connolly and Hill, 1991; Itokawa et al., 1991; Mensah et al., 1992; Mdee et al., 1998; Reina et al., 2002), and hence the corresponding structures were accordingly deduced based on the spectral data.

18-Hydroxy-3-deoxyhugorosenone (**3**, $C_{20}H_{30}O_2$ by ESI-FT-ICR-MS) exhibited spectral features comparable to those displayed by **1** (Mdee et al., 1998). However, the ^{1}H and ^{13}C NMR spectra of **3** (Table 1) lacked signals due to the C-3 carbinol group found in compound **1**. Instead the spectra consisted of signals due to an isolated diastereotopic methylene group (δ_H 2.71 and 2.76, each *d*, $J = 12$ Hz and δ_C 38.68, HMQC), the low field position of the ^{1}H NMR resonances indicating that the methylene unit was adjacent to an unsaturated system. Furthermore, the ^{1}H NMR spectrum displated a signal due to a deshielded vinylic proton appearing at δ 6.09 (*d*, $J = 2.74$ Hz) that only suffered long range coupling (H/H COSY, HMBC) with another methine proton (δ_H 3.02, *ddd*, $J = 11.72$, 2.74, 2.56 Hz). These spectral features suggested that the former proton was isolated, the low field position indicating the proton's close proximity to the carbonyl group (IR, ν_{max} 1717 cm^{-1}; ^{13}C NMR, $\delta_{CO} = 200.47$ ppm). Considering a double bond at C-1(10) and a C-2 carbonyl group, as in compound **1**, the above spectral features were consistent with C-3 constituting the diastereotopic methylene unit in **3**, as further confirmed by H/H COSY and HMBC C/H interactions, which also established the remaining part of structure **3**.

The structure for hugorosenol (**4**, $C_{20}H_{32}O_2$ by ESI-FT-ICR-MS) was deduced based on analysis of spectral data, which were also comparable with those reported for **1**, except that instead of one carbinol there were two such units in **4** (^{1}H and ^{13}C NMR, Table 1), and that the compound lacked a carbonyl group. Considering the oxygenation pattern in compound **1** it was concluded that the C-2 carbonyl group in **1** was transformed into a carbinol unit in **4**, as further confirmed from H/H and C/H interactions observed in the COSY and HMBC spectra. The $J_{1,2}$ and

Table 1
^{1}H and ^{13}C NMR spectral data for 18-hydroxy-3-deoxyhugorosenone (**3**) and hugorosenol (**4**)

H/C	**3**			**4**		
	δ_H	*J* (Hz)	δ_C	δ_H	*J* (Hz)	δ_C
1	6.09	*d*, 2.74	122.3	5.71	*dd*, 4.7, 2.5	118.0
2	–	–	200.5	4.17	*m*	75.8
3_{ax}	2.71	*d*, 12	38.7	3.36	*dd*, 8.4, 5.6	66.8
3_{eq}	2.76	*d*, 12				
4			47.8			36.9
5	3.02	*dd*, 11.72, 2.74, 2.56	37.7	2.11	*dd*[*]	43.9
6_{ax}	1.45	*m*	17.4	1.41	*m*	18.6
6_{eq}	2.02	[a]		1.75	*m*	
7_{ax}	1.32	[a]	24.7	1.28	[*]	25.5
7_{eq}	1.78	[a]		1.62	[*]	
8	2.03	[a]	30.8	1.70	[*]	31.2
9			39.7			37.3
10			175.5			153.4
11_{ax}	1.51	[a]	34.2	1.47	*m*	35.3
11_{eq}	1.77	[a]		1.75	*m*	
12_{ax}	1.25	[a]	41.9	1.31	*m*	32.8
12_{eq}				1.52	*m*	
13			36.1			36.3
14_{ax}	1.36	[a]	32.5	1.16	*m*	39.9
14_{eq}	1.54			1.21	*m*	
15	5.82	*dd*, 17.56, 10.79	150.5	5.82	*dd*, 17.57, 10.8	150.8
16_{ax}	4.94	*dd*, 17.57, 1.28	109.2	4.86	*dd*, 10.61, 1.28	108.8
16_{eq}	4.86	*dd*, 10.79, 1.28		4.93	*dd*, 17.56, 1.28	
17	0.98	*s*	22.2	0.97	*s*	22.3
18a	3.27	*d*, 10.98	68.5	1.04	*s*	25.6
18b	3.65	*d*, 10.98				
19	0.77	*s*	16.2	0.76	*s*	15.6
20	1.04	*s*	18.9	0.97	*s*	20.2
3-OH				2.72	*d*, 8.4	

[a] Signals not resolved.

$J_{2,3}$ values (4.7 and 5.6 Hz, respectively) observed in the ^{1}H NMR spectrum suggested the H-1/H-2 and H-2/H-3 dihedral angles to be about 45° and 60°, respectively (Becker, 1980). This established the $\alpha_{(equatorial)}$ and $\alpha_{(axial)}$ configuration for H-2 and H-3, respectively, the α stereochemistry for H-3 being further indicated by the presence of H-3/H-5 NOE interactions.

The spectral data for the bicyclic sesquiterpenoid hugonianene B (**5**) were comparable with those reported for himachalenoids (Shankaranarayan et al., 1977; Cane, 1999), particularly being very similar to spectral properties of 4α-methoxy-5,9-oxahimachal-9-ene [hugonianene A (**10**), Baraza et al., in press]. However, unlike hugonianene A, compound **5** exhibited ^{13}C NMR signals due to two carbinol C atoms, one of which bearing a methoxyl group ($\delta_H = 3.16$, 3H, *s*; 3.65, 1H, *dd*, $J = 5.1$ and 1.1 Hz and $\delta_C = 57.5$, 83.4 ppm). The H/H COSY and HMBC C/H interactions established the position of the methoxy and the quaternary, hydroxylated carbons at C-4 and the bridgehead C-5, respectively. The relative stereochemistry at C-3 and C-4 was assigned by considering the magnitude of the $J_{3,4}$ value of 5.1 Hz, which requires that H-3 and H-4 are $cis_{axial/equatorial}$ (dihedral angle of *ca.* 60°, Becker, 1980), hence the 5-OH group assuming a β configuration (molecular models). This stereochemical configuration would account for the observed H-1/H-4 coupling ($^5J_{H\text{-}1,H\text{-}4} =$ 1.1 Hz) and the significant ^{13}C NMR deshielding of C-4 as compared to C-5.

Attempts to determine the absolute configuration at C-5 of compound **5** by NMR experiments was unsuccessful, due to paucity of the available sample. Furthermore, sesquiterpene **5** could have been an artefact of the sesquiterpenoid **10** that we recently reported from *Hugonia busseana* (Baraza et al., in press). However, compound **5** was never detected in crude samples from *H. busseana*. Likewise sesquiterpenoid **10** was not detected in crude extracts from *H. castaneifolia*. Furthermore, compound **10** was found to be quite stable, not having been transformed to **5** when let to stand either in pure form or in chloroform, even for over a week. This therefore suggested that compound **5** was not an artefact that could have been formed from **10** during the extraction process or on storage prior to analysis.

Analysis of spectroscopic data established structure **6** for the long chain aldehyde, 2-hydroxyhenpentacont-2-enal ($C_{51}H_{100}O_2$ by HRMS). The presence of an enolyl α,β-unsaturated aldehyde unit was indicated by considering characteristic patterns in the UV (λ_{max} 274 and 317 nm), IR (ν_{max} 3445, 1684, 1635, 1471 and 719 cm^{-1}) and NMR ($\delta_{=CH}$ 6.43, *t*, $J = 7.5$ Hz; $\delta_{CH=O}$ 9.35, *s*; δ_C 143.7, 155.0 and 195.2) spectra, the low field position of the ^{1}H NMR signal at δ 6.43 indicating the corresponding vinyl proton to be within the α,β-unsaturated carbonyl system.

Generally, unsaturated 3-hydroxy or 3-oxo fatty acids, or polyketides are biochemically very commonly formed as quite stable metabolites, but not the related 2-hydroxy or 2-oxo compounds. Thus, previously, 3-hydroxy C_{22}–C_{28} long chain polyketide aldehydes were reported from the leaf cuticular waxes of *Ricinus communis* (Vermeer et al., 2003). However, so far partially degraded polyketides similar to **6** have not been reported. Compound **6** having the enolic group adjacent to the aldehydic carbonyl would be rather unstable, undergoing tautomerism to give the more stable keto form **6a**. However, although compound **6** was found to undergo decomposition upon storage even in solution, the keto form **6a** was never detected, even as a contaminant of pure **6**.

These and previous results (Mdee et al., 1998; Baraza et al., in press) demonstrate the ability of East African *Hugonia* species to accumulate rosane-type diterpenoids. Surprisingly however, lignans and alkaloids have been reported as the only main constituents of *Hugonia* species found outside East Africa (Broomhead and Dewick, 1990; Konuklugi, 1996; Ikhiri, 1987).

Compounds **1**–**3** and **7** exhibited antifungal activity against *Cladosporium cucumericum* Ell. et Arth., while the di-podocarpanoids **8** and **9** were only weakly active, the rest of the isolated compounds being inactive. The antifungal results are given in Table 2, in which a larger area correlates with higher activity. However, such direct correlation is valid only for compounds of similar silica gel/water diffusion values, a factor that was not investigated in these studies. Therefore, these results are only qualitative. In anticipation of the low abundance of the active constituents, the crude extracts were not assayed at lower concentration values while due to paucity of the available

Table 2
Fungal (*Cladosporium cucumericum* Ell. et Arth.) inhibition zones (mm^2) for compounds **1**, **2**, **5** and **7**, and H. castaneifolia crude extracts[a]

Amount (μg)	1	2	3	7	9	Hex Extr	CH_2Cl_2 Extr
6.25	0	7.07	0	0	0	NT	NT
12.5	3.14	19.63	0	8.56	0	NT	NT
25	28.26	38.47	0	19.63	0	NT	NT
50	38.47	63.59	12.56	38.47	7.07	19.63	0
100	50.24	78.50	38.47	63.58	19.63	28.26	3.14
200	63.59	153.86	NT	NT	38.47	19.63	19.63
400	NT	NT	NT	NT	NT	78.50	78.50

[a] Hex Extr = *n*-hexane extract; CH_2Cl_2 Extr = dichloromethane extract; NT = Not tested; activity of Benomyl (used as the standard antifungal agent) = 0, 0, 19.63 and 28.26 mm^2 at 6.25, 12.5, 25.0 and 50.0 ng, respectively.

samples some of the compounds were not available for testing at higher concentration levels. The results suggest that some of the rosane diterpenoids occurring in *H. castaneifolia* may be produced by this plant species as phytoalexins, as previously reported for similar compounds isolated from rice plants (Cartwright et al., 1981; Song and Goodman, 2001).

The crude *n*-hexane and dichloromethane extracts showed larvicidal activity against *Anopheles gambiae* mosquito larvae (LC_{50} values of 0.3028 and 0.0986 mg/ml at 24 and 48 h exposure). However, of the tested compounds **1**, **2**, **4** and tetracosyl-(*E*)-ferrulate, only hugorosenone (**1**) showed any appreciable activity ($LC_{50} = 0.3028$, 0.0674 and 0.0582 mg/ml at 24, 48 and 72 h exposure time, respectively).

3. Experimental

3.1. General remarks

Column chromatography on silica gel 60 (0.063–0.200 mm, Merck); TLC on silica gel 60 F_{254} (Merck) pre-coated plates; visualization: UV–Vis and anisaldehyde spray reagent (Stahl, 1969); prep and analytical HPLC on a Merck-Hitachi L-6250 low-pressure gradient pump with an L-4250 UV detector and a Lichrosper 100-RP 18 column (7 μm, 250 × 10 mm, Merck); IR spectra in $CHCl_3$ or KBr; UV spectra in MeOH; specific rotation $CHCl_3$; 1D NMR spectra 300 MHz (^1H) and 75 MHz (^{13}C); 2D NMR (HMQC, HMBC, COSY, ROESY) at 500 MHz (^1H), and inverse techniques for HMQC and HMBC; chemical shifts in ppm referenced to the internal standard TMS ($\delta = 0$) for ^1H and $CDCl_3$ ($\delta = 77.0$ ppm) for ^{13}C NMR. Electrospray ionisation fourier transform ion cyclotron resonance mass spectra (ESI-FT-ICR-MS) at 7.0 T, RF-only hexapole ion guide and an external electrospray ion source, sample solutions introduced continuously via a syringe pump, flow rate 120 μl/h, EIMS at 70 eV with direct injection.

3.2. Plant material

The root bark of *Hugonia castaneifolia* was collected from Pugu forest, about 25 km west of Dar es Salaam, Tanzania in December 1996 and 2002. The plant species was identified at the Herbarium of the Botany Department, University of Dar es Salaam where a voucher specimen is preserved (FMM 822).

3.3. Extraction and isolation

Air-dried and powdered root bark (500 g) was soaked (48 h) in *n*-hexane and CH_2Cl_2 at room temp (*ca.* 26–30 °C). The conc. crude extracts were fractionated by vacuum liquid chromatography (VLC) over silica gel (petrol/EtOAc gradient). The *n*-hexane extract (5.5 g) after VLC, CC on silica gel [petrol/EtOAc, (9:1 v/v)] and subsequent CC on Sephadex® LH-20 ($CHCl_3$/MeOH, 1:1 v/v) and then recrystallisation (MeOH) yielded **6** (5.3 mg). Repeated CC on silica gel of the remaining VLC fractions gave **1** (154 mg), tetracosyl-(*E*)-ferrulate (14 mg), **7** (18 mg), **2** (5 mg), **3** (4.5 mg) and **8** (28 mg) and trace amounts of **9**. Combined VLC separation of the CH_2Cl_2 extract from the first collection and repeated CC afforded **5** (9.8 mg) and **8** (12 mg). VLC workup of the CH_2Cl_2 extract from the second collection gave four fractions (F1–F4) which after repeated CC on silica gel (petrol/EtOAc, 9:1 to 1:1 v/v) and Sephadex® LH-20 ($CHCl_3$/MeOH, 1:1 v/v) yielded further amounts of **1** (256 mg from F2), **4** (24 mg from F2), caryophyllene oxide (6 mg, from F2 and F3), **7** (8.5 mg from F3), **4** (21 mg from F3), **2** (4.5 mg from F3), **8** (68 mg from F4), and **9** (20 mg from F4). HPLC analysis of VLC fraction F1 [H_2O (solvent A) and CH_3CN (solvent B)] over a linear gradient (0–40 min, 10–100% B; flow rate 1 ml/min) and comparison with authentic samples enabled identification of compounds **1**–**4** in that fraction.

3.3.1. 18-Hydroxy-3-deoxyhugorosenone [18-hydroxy-2-oxorosa-1(10),15-diene] (3)

White gum; yield, 4.5 mg; anisaldehyde-blue; IR, ν_{max}($CHCl_3$) 3638, 3024, 2927, 2854, 1717, 1655, 1609, 1465 and 1379 cm^{-1}; ^1H and ^{13}C NMR (Table 1); EIMS, *m/z* (% rel. int) 302 ($[M]^+$, 40), 286 (15), 271 (100), 252 (20), 241 (8) and 121 (18); ESI-FT-ICR-MS, *m/z* 325.23013 $[M+Na]^+$, Calc. for $C_{20}H_{30}O_2Na$: 325.214350.

3.3.2. Hugorosenol [1(10),15-rosadiene-2β,3β-diol] (4)

White needles (EtOAc/*n*-hexane); m.p. 139–141 °C; yield, 45 mg; $[\alpha]_D^{23}$ −13.37° ($CHCl_3$; c 0.19); anisaldehyde–blue; IR, ν_{max} (KBr) 3402, 3078, 2972, 2940, 2875, 1635, 1559, 1506, 1465, 1376, 1068 and 1035 cm^{-1}; UV, λmax (MeOH) nm (log ε) 298 (5.0) and 270 (1.69); EIMS, *m/z* (% rel. int.) 304 ($[M]^+$, 100), 281 (8), 271 (12), 232 (70), 217 (15), 149 (20), 121 (25), 107 (30), 96 (65) and 81 (14); ESI-FT-ICR-MS, *m/z* 327.23042 $[M+Na]^+$, Calc. for $C_{20}H_{32}O_2Na$: 327.2294514; ^1H and ^{13}C NMR (Table 1).

3.3.3. Hugonianene B (4α-methoxyhimachal-10-en-5β-ol) (5)

Gum; yield, 9.8 mg; $[\alpha]_D^{25}$ −55° ($CHCl_3$; c 0.1); anisaldehyde–blue; IR, ν_{max} 3500, 2852, 2842, 1058 and 978 cm^{-1}; ^1H NMR: δ 0.63 (3H, *d*, $J = 6.5$ Hz, H-15), 0.80 (2H, *m*, H-7), 0.90 (3H, *s*, H-12), 0.95 (3H, *s*, H-13), 1.35 (1H, *m*, H-8_{ax}), 1.62 (2H, *m*, H-2), 1.55 (3H, *m*, H-14), 1.75 (1H, *m*, H-8_{eq}), 1.8 (1H, *dm*, H-1_{ax}), 2.0 (1H, *m*, H-9_{ax}), 2.2 (1H, *m*, H-9_{eq}), 2.71 (1H, *dm*, 13 Hz, H-1_{eq}), 3.16 (3H, *s*, CH_3) and 3.65 (1H, *dd*, $J = 5.1$, 1.1, H-4); ^{13}C NMR, δ 141.4 (C-11), 125.5 (C-10), 83.4 (C-4), 65.0 (C-5), 57.5 (OCH_3), 48.5 (C-1), 42.8 (C-6), 35.3 (C-3), 30.2, −27.9 (C-2, 7, 8, 9 and 14), 20.3 (C-13), 18.8 (C-12) and 14.5 (C-15); EIMS, *m/z* (% rel. int)] 252 ($[M]^+$, <5), 234 ($[M-H_2O]^+$, 70), 203 (30), 202 (100) and 159 (85); ESI-FT-ICR-MS, *m/z* 275.21002 $[M+Na]^+$, Calc. for $C_{16}H_{28}O_2Na$: 275.198700.

3.3.4. 2-Hydroxyhenpentacont-2-enal (6)

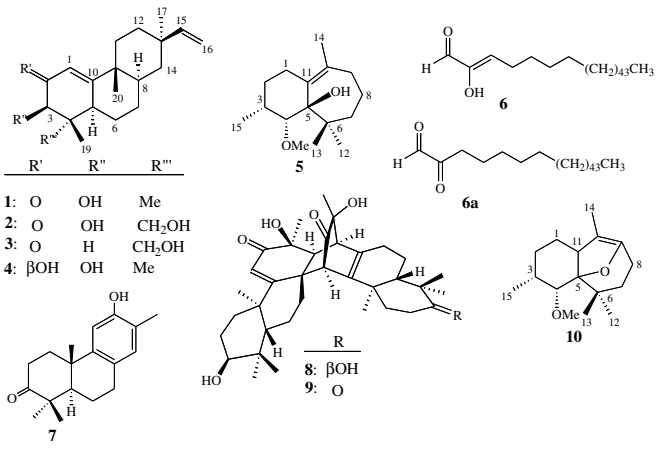
White amorphous solid (MeOH); m.p. 39–40 °C; yield, 5.3 mg; anisaldehyde–blue; UV, λ_{max} 274 and 317 nm; IR, ν_{max} (KBr) 3445, 2915, 2848, 1684, 1635, 1471 and 719 cm^{-1}; ^1H NMR, δ 9.35 (1H, *s*, H-1), 6.43 (1H, *t*, $J = 7.5$ Hz, H-3), 2.37 (2H, *m*, H-4) and 1.25 (*br s*, 92H, long chain) and ^{13}C NMR, δ 195.2 (C-1), 155.0 (C-2), 143.7 (C-3), 32.0 (C-4), 29.7–29.4 [long chain $(CH_2)_{46}$] and 14.2 (CH_3); EIMS, *m/z* (% rel. int.) 744 ($[M]^+$, ~5), 687 (100), 659 (80), 631 (35), 603 (15), 419 (8), 392 (15), 378 (25), 363 (20), 349 (15), 135 (18), 98 (55) and 83 (48); HR-EIMS, *m/z* 744.80023 ($[M]^+$, Calc. for $C_{51}H_{100}O_2$: 744.772333).

3.4. Antifungal assay

A dilution series of the crude extracts (*n*-hexane and CH_2Cl_2) as well as the isolated compounds in $CHCl_3$ in the range from 6.25 to 400 μg was spotted on 0.5 mm thin layer silica gel plates and sprayed with an aqueous, nutritive spore suspension of the phytopathogenic fungus *Cladosporium cucumerinum* Ell. et Arth (Gottstein et al., 1982) using Benomyl as the standard antifungal agent. After two days in a wet chamber (>95% humidity), the plates were overgrown with a dark grey coloured mycelium. Areas with sufficient antifungal substance were recognizable as white spots (inhibition area). A relative quantitative estimation was deduced from the size and intensity of the spots according to Gottstein et al. (1982).

3.5. Mosquito larvicidal assay

This was carried out as previously described (Joseph et al., 2004).



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