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Nor-hopanes from *Zanha africana* root bark with toxicity to bruchid beetles



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ABSTRACT

Zanha africana (Radlk.) Exell (Sapindaceae) root bark is used by farmers throughout sub-Saharan Africa to protect stored grain from bruchid beetles, such as *Callosobruchus maculatus*. Chloroform, methanol and water extracts of *Z. africana* root bark inhibited oviposition and caused significantly higher mortality of *C. maculatus* at a rate of application equivalent to that applied by farmers compared to control insects. The chloroform extract contained *nor*-hopanes rarely found in plants of which seven were isolated, one of which was previously known. Two of the most abundant *nor*-hopanes 3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-21 α H-24-*nor*hopa-4(23),22(29)-diene and 3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-24-*nor*hopa-4(23),17(21)-diene were toxic to and reduced oviposition of *C. maculatus* in a dose dependent manner. *Z. africana* root bark is rich in insecticidal compounds that account for its effective use by smallholder farmers as an alternative to conventional insecticides.

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1. Introduction

Zanha africana (Radlk.) Exell is a medium sized tree belonging to the Sapindaceae (Flora Zambesiaca) and occurs in the African savannah and distributed from Kenya southwards through Tanzania, Malawi, Mozambique, Zambia, Zimbabwe, southern Angola and Namibia (Beentje, 1994; Swanepoel, 2013). *Zanha* species have cultural importance across the range. For example, *Zanha golunguensis* is a source of medicine (Bruschi et al., 2011) with activity reported in bark against trypanosomiasis (Nibret et al., 2010), bacterial pathogens (Kambizi and Afolayan, 2001) and fungi (Fabry et al., 1996), and also has anti-inflammatory activity (Recio et al., 1995). *Z. africana* is rich in oleanane type saponins based on the zanhagenic triterpene skeleton (Kapundu et al., 1992)

including zanhasaponins A, B and C isolated from the root bark which reportedly account for anti-inflammatory properties (Cuellar et al., 1997a,b). More recently, the structurally related compounds zanhasaponins D–H have also been reported from root bark of *Z. golunguensis*, the only other species in the genus growing in Africa (Lavaud et al., 2015).

Smallholder farmers in Tanzania use the root bark of *Z. africana* to protect stored grain from stored product pests (Mkoga et al., 2004) by pounding the stripped bark to a powder and admixing with their grain. The potential livelihood impact of wild, locally available plants in pest control is compelling if they can be sustainably sourced, particularly for poorly-resourced small-scale farmers (Grzywacz et al., 2014; Isman, 2006). The aim of this study was to analyse the chemistry of *Z. africana* root bark and identify components that might be responsible for any biological activity against insects. Understanding the chemical basis of activity in pesticidal plants provides tools necessary to explain temporal and spatial variation in efficacy (Belmain et al., 2012), inform about the occurrence of chemotypes (Stevenson et al., 2012), and enable the development of optimized field application (Stevenson et al., 2009).

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In this paper, the identity of seven *nor*-hopanes from the root bark of *Z. africana* was determined of which six are reported for the first time. These components explain, at least in part, the bio-efficacy of the root bark of *Z. africana* in protecting stored cowpeas from bruchid damage in smallholder farm stores.

2. Results and discussion

2.1. Identification of *nor*-hopanes in *Z. africana*

The chloroform extract of *Z. africana* root bark was shown to be toxic to a bruchid beetle, *Callosobruchus maculatus* (L.), in bioassays described in detail below (Sections 2.2 and 3.1). This extract was analysed using LC-UV-MS/MS and indicated the presence of numerous non-polar peaks with similar UV spectra. Seven compounds, **1–7** (Fig. 1), were isolated using semi-preparative HPLC and characterized using spectroscopic techniques.

Full assignment of the ^1H and ^{13}C NMR spectra of **1** in CDCl_3 was obtained using COSY, HSQC and HMBC data (Tables 1 and 2). The ^{13}C NMR assignments of **1** showed a good match with those for 3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene (Chávez et al., 1997). Good agreement was also found between the ^1H NMR assignments and a partial dataset given by the latter authors, with the exception of the assignments of H-9 and H-13 which required revision (Table 1). A second NMR dataset for **1** was acquired in $\text{MeOH-}d_4$ because of the improved resolution of the multiplet structure of several key resonances including H-3 and H-6. A series of 1D site selective ROE experiments indicated that **1** had the same relative configuration as the published structure (Fig. 1). In particular, the α -configuration of H-21 was confirmed by an ROE correlation with 28-Me. Other key ROE correlations were between 28-Me and 27-Me, 27-Me and H-7 (confirming the β -configuration of the 7-(4-hydroxybenzoyl)oxy group), H-7 and H-5, H-6 and H-5 (confirming the β -configuration of the 6-OH group), H-5 and H-3 (confirming the β -configuration of the 3-OH group) and 25-Me and 26-Me. The optical rotation for **1** of $\alpha_D = +45.7$ (c 0.54, MeOH) had the same sign as the literature value of $\alpha_D = +10$ (c 0.79, CHCl_3).

The molecular formula of **2** established by HRESIMS as $\text{C}_{36}\text{H}_{50}\text{O}_6$ differed from that of **1** by the inclusion of one additional oxygen atom. Full assignment of the ^1H and ^{13}C NMR spectra of **2** was carried out in both CDCl_3 and $\text{MeOH-}d_4$. Comparison of the latter with the analogous assignments for **1** indicated that **2** possessed an oxygenated methine in place of a methylene group. In the COSY spectrum (CDCl_3), the oxygenated methine (δ_{H} 4.30) correlated with H-9 (δ_{H} 1.65) and 12- CH_2 (δ_{H} 1.81 and 1.59). Similarly, in the HMBC spectrum acquired in CDCl_3 , correlations were observed from δ_{H} 4.30 to C-8 (δ_{C} 48.4), C-9 (δ_{C} 54.2), and C-10 (δ_{C} 39.8). The additional hydroxyl group was therefore located at C-11. NOE connectivities observed between both 25-Me and 26-Me with H-11 indicated that this hydrogen atom was β -oriented. The significant downfield shift ($\Delta\delta + 1.06$ ppm) experienced by H-1 β (δ_{H} 2.89 in CDCl_3) was also consistent with an α -configuration for 11-OH (Isaka et al., 2011). Compound **2** was therefore 3 β ,6 β ,11 α -trihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene.

The ^1H NMR spectrum of **3** was similar to that of **2** with the exception that H-11 (δ_{H} 5.48 in $\text{MeOH-}d_4$) showed a significant downfield shift ($\Delta\delta + 1.28$ ppm) and a 3H singlet at δ_{H} 2.03 was observed corresponding to an acetyl group (δ_{C} 172.1 and 22.1). In the HMBC spectrum, H-11 correlated with the acetyl carbonyl group at δ_{C} 172.1 and also with C-9 (δ_{C} 52.9). In the COSY spectrum, H-11 correlated with both H-9 and 12- CH_2 as expected. The magnitude of the coupling constant $J_{9,11}$ of 11.3 Hz indicated a diaxial relationship between these hydrogen atoms such that H-11 was

β -oriented. Compound **3** was therefore 11 α -acetoxy-3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene.

The main difference between the ^1H NMR spectra of **4** and **2** was that the former contained resonances corresponding to two 4-hydroxybenzoyl groups rather than one. The first set of resonances was assigned to the 4-hydroxybenzoyl group at 7-OH. The second was placed at 11-OH on the basis of the large downfield shift ($\Delta\delta + 1.56$ ppm) experienced by H-11 (δ_{H} 5.76) and the long-range correlation between this proton and the remaining 4-hydroxybenzoyl carbonyl at δ_{C} 167.3. As expected, H-11 correlated with H-9 and 12- CH_2 in the COSY spectrum. In common with **3**, $J_{9,11}$ for **4** was also 11.3 Hz, confirming a diaxial relationship between H-9 and H-11 with the latter β -oriented. Thus compound **4** was 3 β ,6 β -dihydroxy-7 β ,11 α -di[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene.

A full set of ^1H and ^{13}C NMR resonance assignments was obtained for **5** using COSY, HSQC and HMBC data. Compound **5** was isomeric with **1** and could be readily identified as a 24-norhopadiene derivative. The difference between the two compounds resided in the structure of the E-ring. In the case of **5**, the E-ring was a fused cyclopentene with an isopropyl group at C-21, whereas **1** featured an isopropylidene group attached to C-21 of a fused cyclopentane moiety. The multiplet structure and J -values for H-3, H-6, and H-7 ($\text{MeOH-}d_4$) were similar for **5** and **1** indicating that the configurations of these atoms were conserved between the two compounds. Thus **5** was 3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene.

The molecular formula of compound **6** differed from that of **5** by the inclusion of one additional oxygen atom, and it was also isomeric with **2**. Analysis of its ^1H and ^{13}C NMR spectra indicated that **6** was the 11 α -hydroxyl derivative of **5**. Thus in the HMBC spectrum acquired in CDCl_3 , H-11 (δ_{H} 4.25) correlated with C-8 (δ_{C} 48.5), C-9 (δ_{C} 54.5), C-10 (δ_{C} 39.8) and C-12 (δ_{C} 36.6). Similarly in the COSY spectrum, H-11 correlated with H-9 and 12- CH_2 . The relative configuration of **6** was examined in a series of 1D site selective ROE experiments (Fig. 2). In particular, H-11 correlated with both 25-Me and 26-Me, as was also found with the isomeric **2**, allowing the β -orientation to be assigned. Thus **6** was 3 β ,6 β ,11 α -trihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene.

Comparison of the ^1H NMR spectra of **7** with **6** indicated that 2- CH_2 , H-5 and 23- CH_2 were all downfield shifted, and the doublet resonance of H-3 was lacking. In the ^{13}C NMR spectrum, there were 3 rather than 4 resonances attributable to oxygenated methines and a new resonance at δ_{C} 204.8 assigned to a carbonyl group. The location of the latter was readily established as C-3 from HMBC data, with correlations from 1- CH_2 , 2- CH_2 , H-5, and 23- CH_2 to δ_{C} 204.8 detected in the spectrum. Compound **7** was thus 6 β ,11 α -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-3-oxo-24-norhopa-4(23),17(21)-diene.

2.2. Biological evaluation of compounds from *Z. africana* against bruchids

Water, methanol and chloroform extracts of *Z. africana* root bark (10% w/v) significantly reduced the number of eggs laid per female bruchid when compared to the solvent control both prior to and after the exposure to cowpeas (*Vigna unguiculata* L. (Walp)) (Table 4 and Fig. 2). None of the treatments at equivalent concentrations were more toxic than rotenone, the positive control. Water and chloroform extracts assayed as a 10% w/v extract of dry root bark also increased mortality of bruchids over a six day exposure period (Table 4). Prior to the addition of the cowpeas, the females actively probed on vials for suitable oviposition sites and left visible marks in the extract residues on the vial surface indicative of

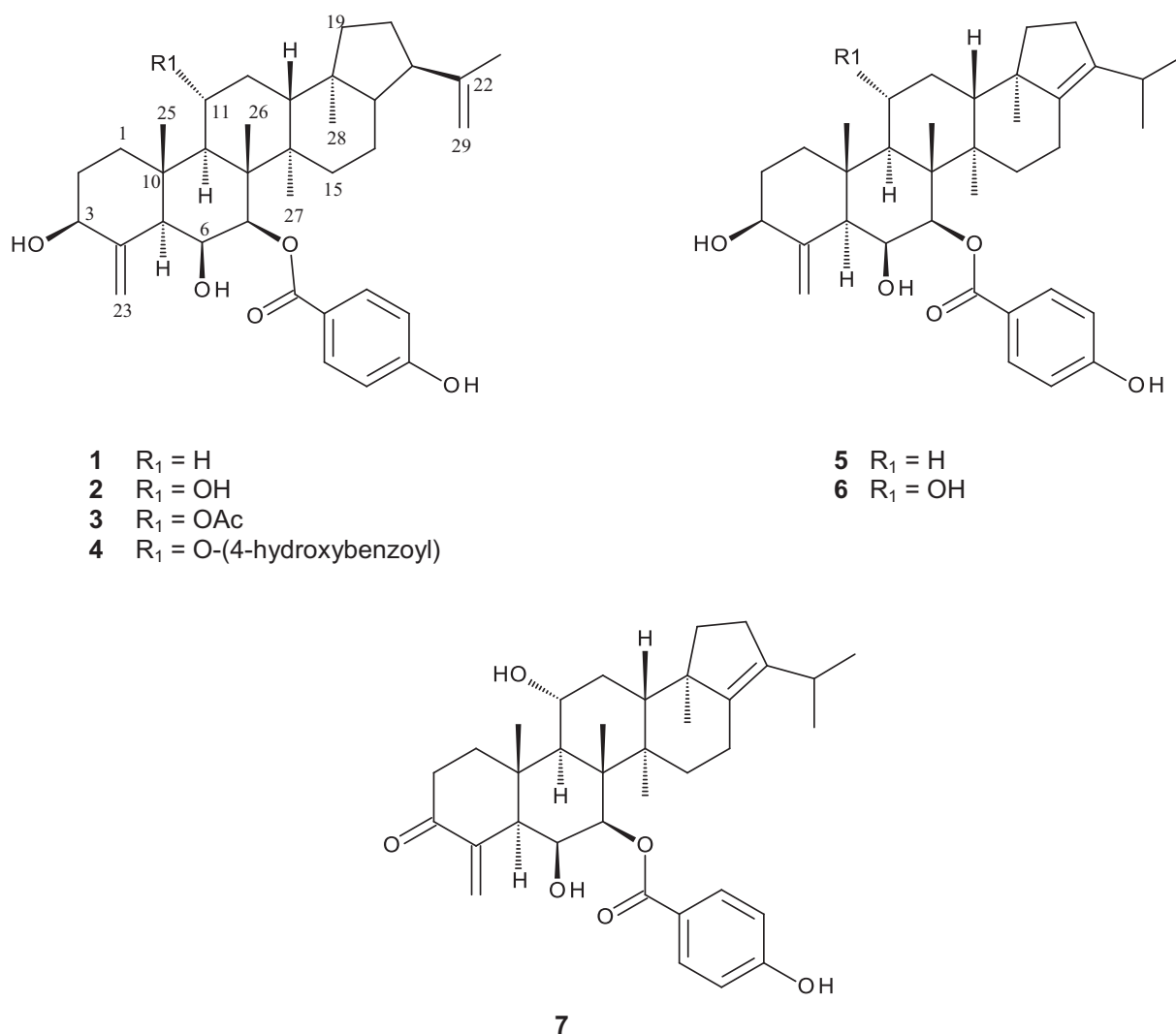


Fig. 1. Nor-hopanes isolated from *Zanha africana* root-bark (see text for detail).

this behaviour; they also deposited eggs. Probing is part of a sequence of behaviours leading to oviposition (Parr et al., 1996, 1998) and provides a route of absorption of toxins, where present, via ovipositors. Females should have laid eggs on the cowpeas when given the opportunity, but in the presence of all extracts they laid significantly fewer eggs compared with the solvent control (Table 4). Similarly, a significantly reduced oviposition was recorded from insects in the presence of **1** and **5** at all concentrations evaluated (10, 100, 1000 ppm) in comparison to the solvent control and this effect was not significantly different to rotenone, the positive control (Table 4 and Fig. 2). The effects of the two nor-hopanes on bruchid mortality were influenced by concentration and exposure period. Mortality observed after 1, 2, 3 and 6 days exposure were described by linear regression, with significantly increasing mortality observed with increasing exposure period and increasing concentration (S1). Although the addition of untreated beans after 72 h provided a refuge from exposure to the extract and compounds, they did not prevent further mortality of insects. In fact, the mortality continued to increase after 72 h (S1). These data suggest that the nor-hopanes are toxic to bruchids but are less toxic than other highly potent plant compounds such as rotenone and deguelin identified in *Tephrosia vogelii* that is used as a natural pesticide in East and Southern Africa (Belmain et al., 2012).

Hopanes, while rare, are previously known from plants, for example, from *Megacodon stylophorus* (Gentianaceae) (Liu et al., 2014) and are elsewhere reported to be insecticidal compounds produced by entomopathogenic fungi (Isaka et al., 2011). Nor-hopanes, however, have previously been of interest primarily due to their occurrence in crude oil albeit in highly reduced form (Prince et al., 1994) rather than the oxidised and benzoyloxy substituted products reported here and have not been reported before as insecticidal compounds. While **1** and **5** were not potent insecticidal compounds on their own compared to rotenone, the positive control, it is likely that they contribute to the insecticidal effects of *Z. africana* preparations that are used by farmers. *Zanha* species are also rich in saponins (Cuellar et al., 1997a,b; Lavaud et al., 2015) which occur widely in other plants used for control of a agricultural pests (Jain and Tripathi, 1991; Mongalo et al., 2015; Nozzolillo et al., 1997; Shinoda et al., 2002) and, in some cases, also explain, biological activities against storage pests including bruchids activity (Stevenson et al., 2009). Thus further work may reveal additional value of saponins of this species but the present work provides scientific evidence that at least partially underpins the use of *Z. africana* by resource-poor farmers based on the occurrence nor-hopanes that reduce the survival and oviposition of storage pests. The commercial potential of plant materials as pesticides is constrained by regulatory hurdles and plants such as *Z. africana*

Table 1
¹H NMR spectroscopic data (δ ¹H, J in Hz) for **1–4** in ¹³CDCl₃ or MeOD.

Atom	*1	1	*2	2	3	4
1	1.83 m, 1.12 m	1.81 m, 1.13 m	2.89 m, 1.19 m	2.89 dt (13.7, 3.6), 1.21 m	2.33 dt (13.6, 3.7), 1.32 m	2.33 dt (13.5, 3.5), 1.42 m
2	1.99 m, 1.49 m	1.88 m, 1.50 m	1.94 m, 1.49 m	1.82 m, 1.48 m	1.85 m, 1.45 m	1.72 m, 1.31 m
3	3.98 br m	3.93 dd (11.6, 5.3)	3.98 br m	3.91 br m	3.93 dd (11.8, 5.4)	3.92 dd (11.8, 5.3)
5	1.79 m	1.76 m	1.83 m	1.78 m	1.83 m	1.87 m
6	4.43 br m	4.38 dd (3.8, 2.1)	4.37 dd (3.6, 2.0)	4.31 dd (3.7, 2.0)	4.32 dd (3.8, 2.0)	4.34 dd (3.8, 1.9)
7	5.30 d (3.9)	5.17 d (3.8)	5.26 d (3.6)	5.12 d (3.7)	5.15 d (3.8)	5.19 d (3.8)
9	1.56 m	1.60 m	1.65 m	1.68 m	2.01 d (11.3)	2.16 d (11.3)
11	1.74 m, 1.57 m	1.77 m, 1.60 m	4.30 m	4.20 m	5.48 dt (11.0, 5.3)	5.76 dt (10.8, 5.5)
12	1.51 m (2H)	1.55 m (2H)	1.81 m, 1.59 m	1.77 m, 1.69 m	1.79 m, 1.64 m	1.85 m, 1.77 m
13	1.53 m	1.59 m	1.67 m	1.69 m	1.75 m	1.81 m
15	1.55 m, 1.04 m	1.64 m, 1.04 m	1.45 m, 1.06 m	1.55 m, 1.02 m	1.56 m, 1.03 m	1.61 m, 1.06 m
16	1.22 m, 1.09 m	1.13 m (2H)	1.23 m, 1.11 m	1.16 m (2H)	1.17 m, 1.13 m	1.19 m, 1.14 m
17	0.98 m	1.04 m	0.97 m	1.03 m	1.05 m	1.07 m
19	1.53 m, 1.07 m	1.55 m, 1.11 m	1.54 m, 1.09 m	1.55 m, 1.14 m	1.50 m, 1.14 m	1.50 m, 1.16 m
20	1.83 m, 1.44 m	1.84 m, 1.45 m	1.85 m, 1.46 m	1.86 m, 1.47 m	1.85 m, 1.47 m	1.83 m, 1.46 m
21	2.20 m	2.21 m	2.21 m	2.22 m	2.22 m	2.23 m
23	5.36 br m, 5.20 br m	5.34 br m, 5.20 br m	5.40 br m, 5.22 br m	5.39 dd (3.7, 1.8), 5.21 m	5.41 m, 5.22 m	5.40 m, 5.19 m
25-Me	1.06 s	1.05 s	1.27 s	1.24 s	1.10 s	1.11 s
26-Me	1.62 s	1.67 s	1.65 s	1.69 s	1.75 s	1.81 s
27-Me	1.07 s	1.08 s	1.09 s	1.09 s	1.12 s	1.18 s
28-Me	0.68 s	0.71 s	0.70 s	0.74 s	0.72 s	0.73 s
29	4.64 dd (2.5, 1.3), 4.62 dm (2.5)	4.64, 4.60	4.65 dd (2.5, 1.4), 4.62 dm (2.5)	4.64 dd (2.5, 1.4), 4.59 dm (2.5)	4.64 dd (2.4, 1.4), 4.60 m	4.65 dd (2.4, 1.4), 4.60 m
30-Me	1.65 s	1.64 s	1.64 br dd (1.4, 0.6)	1.63 br dd (1.4, 0.6)	1.63 s	1.64 s
7-O-(4-OHBz)						
2,6	7.97 d (8.8)	7.93 d (8.9)	7.95 d (8.8)	7.92 d (8.8)	7.93 d (8.8)	7.94 d (8.8)
3,5	6.89 d (8.8)	6.85 d (8.9)	6.88 d (8.8)	6.84 d (8.8)	6.85 d (8.8)	6.86 d (8.8)
11-OAc						
Me					2.03 s	
11-O-(4-OHBz)						
2,6						7.89 d (8.8)
3,5						6.85 d (8.8)

are unlikely to replace synthetic products (Sola et al., 2014). Isman (2006), however, suggests that the value of pesticidal plants will be most important in developing countries by poorer farmers. But, this requires greater scientific information to help understand how use of pesticidal plants might be optimised. In this respect, the present work provides important knowledge for understanding more about chemical variability, persistence, residues and improving application.

3. Experimental section

3.1. General instrumentation

HRESIMS data were recorded using a Thermo LTQ-Orbitrap XL mass spectrometer linked to a Thermo Accela LC system performing chromatographic separation of 5 μ l injections on a Phenomenex Luna C18(2) column (150 mm \times 3.0 mm i.d., 3 μ m particle size) with a linear mobile phase gradient of MeOH: H₂O containing 0.1% HCO₂H (90% H₂O at t = 0 min to 100% MeOH at t = 20 min). Spectra were recorded in either the positive or negative ion modes at 30,000 resolution.

NMR spectra were acquired in CDCl₃ or MeOH-*d*₄ at 30 °C on either a Bruker 400 (Avance) MHz instrument or a Bruker 700 (Avance II+) MHz instrument equipped with a 5 mm 1H/13C/15N triple-resonance PFG cryoprobe. Standard pulse sequences and parameters were used to obtain one-dimensional ¹H, ¹³C, and site selective NOE or ROE, and two-dimensional gradient-enhanced COSY, HSQC, and HMBC spectra. Chemical shift referencing was carried out using TMS for samples dissolved in CDCl₃ and the internal solvent resonances at δ _H 3.31 and δ _C 49.1 (calibrated to TMS at 0.00 ppm) for samples dissolved in MeOH-*d*₄.

Optical rotation measurements (sodium D line, λ 589 nm, 22 °C) were made using a Perkin–Elmer 141 polarimeter with a 10 cm light path cylindrical cell of 1 ml volume. UV and CD spectra (22 °C, MeOH, 0.25 mg/ml) were acquired on an Applied Photo-physics Ltd., Chirascan spectropolarimeter with the following parameters: 1 nm bandwidth, 1 nm step-size and a 0.5 s instrument time-per-point sampling; 2 mm and 0.5 mm cell path lengths were employed in the wavelength range 500–200 nm and all spectra were solvent baseline subtracted.

3.2. Plant material and extraction

Z. africana was collected from a field site in Kilos, Morogoro, Tanzania and a voucher specimen (Ref SUA - ASHC 1) is deposited at the national herbarium, at Arusha and verified by Dr. E-F. A. Njau. The root bark was removed and air dried in the dark. Root bark from *Z. africana* (155 g) was coarsely ground into pieces (1–5 mm) in a coffee-mill before extraction in *n*-hexane (1.55 L). After 48 h, the extract was filtered using a Büchner funnel and the plant material left in a fume-cupboard to allow any solvent residue to evaporate before adding CHCl₃ (1.55 L). This process was repeated for extracts in MeOH and H₂O. Filtered extracts were dried under vacuum affording extract residues of 0.20 (*n*-hexane), 0.80 (CHCl₃), 35.84 (MeOH) and 10.10 g (H₂O), respectively.

3.3. Analysis and isolation of nor-hopanes

The dried CHCl₃ extract was re-dissolved in MeOH to 50 mg mL⁻¹. An HPLC system consisting of a Waters 2695 separations module linked to a 2996 photodiode array detector (PDAD) were used for visualization and isolation of the *nor*-hopanes. Aliquots (90 μ L) were injected onto a Phenomenex Luna RP18 column

Table 2¹³C NMR spectroscopic data for 1–7 in ¹³CDCl₃ or MeOD.

Atom	*1	1	2	3	4	5	*6	6	7
1	40.4	41.8	43.6	43.6	43.7	41.8	42.4	43.7	41.6
2	32.5	33.2	33.7	33.7	33.7	33.3	32.9	33.7	37.2
3	73.1	74.0	74.1	73.8	73.8	74.0	73.1	74.1	204.8
4	149.1	151.1	151.6	151.3	151.2	151.1	149.6	151.7	146.6
5	50.5	51.6	51.8	51.3	51.2	51.6	50.6	51.9	51.9
6	71.7	72.0	72.0	71.8	71.8	72.2	71.8	72.2	69.8
7	75.5	77.3	77.0	76.6	76.6	77.8	75.4	77.3	77.1
8	46.7	48.2	49.8	50.1	50.2	48.3	48.5	49.9	49.6
9	49.3	50.8	55.3	52.9	53.2	51.3	54.5	55.7	54.7
10	38.4	39.8	41.3	40.8	41.0	39.9	39.8	41.3	38.9
11	21.4	22.7	71.0	74.5	74.8	23.0	70.5	71.1	71.0
12	24.1	25.4	37.0	32.8	32.9	25.6	36.6	36.9	36.9
13	48.1	49.7	48.3	47.9	47.9	50.5	47.4	48.9	49.1
14	44.3	45.6	45.3	45.2	45.2	45.2	43.6	44.9	44.9
15	35.1	37.0	36.9	36.8	36.8	36.1	33.9	35.9	36.0
16	21.4	22.4	22.6	22.5	22.5	21.2	20.2	21.2	21.2
17	53.7	54.9	55.0	55.0	55.0	140.9	138.5	140.4	140.2
18	44.2	45.5	45.3	45.3	45.2	51.2	49.4	50.9	50.9
19	40.6	41.7	41.4	41.3	41.2	43.2	41.7	42.8	42.9
20	27.1	28.3	28.3	28.3	28.3	28.2	27.4	28.4	28.4
21	48.0	49.5	49.4	49.4	49.4	137.3	136.8	137.7	137.7
22	148.0	149.0	149.0	148.8	148.8	27.6	26.3	27.6	27.6
23	105.3	105.9	106.0	106.5	106.4	105.9	105.5	106.0	122.2
25-Me	16.1	16.7	16.7	16.9	17.0	16.7	16.2	16.7	16.5
26-Me	12.7	13.2	13.8	13.9	14.0	12.8	13.2	13.6	13.7
27-Me	17.3	17.9	18.3	18.3	18.3	16.0	15.7	16.2	16.1
28-Me	15.1	15.6	15.4	15.4	15.4	19.4	18.4	19.0	19.1
29	109.6	110.3	110.3	110.5	110.5	22.3	21.8	22.3	22.2
30-Me	19.8	19.8	19.9	19.9	19.9	21.7	21.3	21.7	21.7
7-O-(4-OHBz)									
CO	165.1	167.8	167.8	167.6	167.7	167.6	164.9	167.6	167.4
1	123.3	123.4	123.4	123.2	123.1	123.3	123.1	123.2	123.1
2,6	132.0	133.2	133.1	133.2	133.2	133.2	132.1	133.2	133.2
3,5	115.5	116.3	116.3	116.4	116.4	116.3	115.5	116.3	116.3
4	159.9	163.6	163.7	163.7	163.7	163.6	160.0	163.7	163.7
11-OAc									
CO				172.1					
Me				22.1					
11-O-(4-OHBz)									
CO					167.3				
1					123.1				
2,6					133.1				
3,5					116.4				
4					163.8				

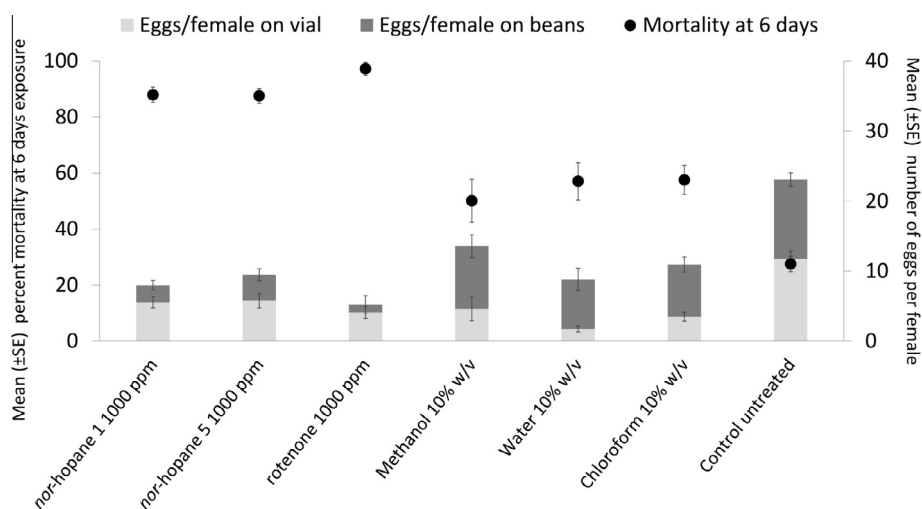
**Fig. 2.** Relationship between bruchid mortality and number of eggs laid per female when exposed for six days to crude extracts and isolated *nor*-hopane compounds from *Zanha africana* root bark and a positive control (rotenone).

Table 3
¹H NMR spectroscopic data (δ ¹H, J in Hz) for **5–7** in ¹³CDCl₃ or MeOD.

Atom	5	6 CDCl ₃	6 MeOD	7
1	1.82 m, 1.16 m	2.90 dt (13.8, 4.0), 1.20 m	2.90 dt (13.6, 3.6), 1.22 m	3.18 ddd (13.6, 6.3, 3.9), 1.58 m
2	1.89 m, 1.50 m	1.94 m, 1.50 m	1.82 m, 1.48 m	2.45 m (2H)
3	3.94 dd (11.7, 5.5)	3.98 dd (11.6, 5.5)	3.92 dd (11.8, 5.5)	
5	1.77 m	1.84 br s	1.80 m	2.45 m
6	4.37 dd (3.6, 2.0)	4.36 d (3.6, 2.0)	4.30 d (3.5, 2.0)	4.40 dd (3.6, 2.3)
7	5.17 d (3.6)	5.27 d (3.6)	5.13 d (3.5)	5.21 d (3.6)
9	1.60 m	1.69 m	1.72 m	1.83 d (10.7)
11	1.78 m, 1.56 m	4.25 dt (10.8, 4.8)	4.14 dt (10.5, 4.4)	4.19 m
12	1.47 m (2H)	1.77 m, 1.50 m	1.73 m, 1.57 m	1.75 m, 1.61 m
13	1.58 m	1.67 m	1.69 m	1.71 m
15	1.50 m, 1.22 m	1.36 m, 1.16 m	1.43 m, 1.18 m	1.46 m, 1.23 m
16	2.08 m, 1.85 m	2.09 m, 1.84 m	2.09 m, 1.87 m	2.10 m, 1.88 m
19	1.70 m, 1.35 m	1.69 m, 1.32 m	1.72 m, 1.39 m	1.73 m, 1.40 m
20	2.20 m, 2.13 m	2.20 m, 2.12 m	2.21 m, 2.16 m	2.23 m, 2.17 m
22	2.58 m	2.54 septet (6.9)	2.57 m	2.58 septet (6.9)
23	5.34 m, 5.19 m	5.40 m, 5.22 m	5.39 m, 5.20 m	6.07 dd (2.5, 1.6), 5.53 dd (2.5, 1.7)
25-Me	1.05 s	1.28 s	1.25 s	1.32 s
26-Me	1.60 s	1.59 s	1.62 s	1.64 s
27-Me	1.18 s	1.19 s	1.19 s	1.21 s
28-Me	0.86 s	0.84 s	0.89 s	0.89 s
29-Me	0.97 d (6.8)	0.95 d (6.9)	0.97 d (6.8)	0.97 d (6.8)
30-Me	0.88 d (6.8)	0.87 d (6.9)	0.88 d (6.8)	0.88 d (6.8)
7-O-(4-OHBz)				
2,6	7.94 d (8.8)	7.96 d (8.9)	7.92 d (8.8)	7.94 d (8.8)
3,5	6.85 d (8.8)	6.89 d (8.9)	6.85 d (8.8)	6.86 d (8.8)

(150 × 10 mm, length × i.d.; 10 μm particle size, maintained at 30 °C) and eluted at 5 mL min⁻¹ using a non-linear gradient (curve = 4) of 50% A: 40% B: 10% C ($t = 0$) to 90% A: 0% B: 10% C ($t = 20$ –30 min) returning to the starting conditions ($t = 31$ min), where A = HPLC-MeOH; B = H₂O and C = 1% HCO₂H in CH₃CN. Automatic collection using a Waters fraction collector (WFC III) yielded five compounds **1** (8.1 mg; $t_r = 12.1$ min), **2** (2.6 mg; $t_r = 7.8$ min), **5** (12.5 mg; $t_r = 12.8$ min), **6** (7.2 mg; $t_r = 8.2$ min), **7** (2.4 mg; $t_r = 8.7$ min) while a sixth peak at $t_r = 9.7$ was subjected to further HPLC using the same method described above, but with CH₃CN in place of MeOH and a non-linear gradient (Waters curve = 3) of 50%A:40%; B 10%C ($t = 0$) to 90%A:0%B:10%C ($t = 20$ –21 min) returning to the original conditions ($t = 22$ min). This procedure yielded two further compounds: **3** (~0.5 mg; $t_r = 5.9$ min) and **4** (~0.5 mg; $t_r = 5.2$ min). All compounds were isolated to a purity of at least 95%.

The optical rotations of **1–3** and **5–7** were individually recorded on a Perkin-Elmer 343 spectrometer. The instrument was calibrated with D (+) sucrose (1.04 g/100 ml, 1 dm, $[\alpha]_{589} = +66.6^\circ$). A 10 cm cylindrical Quartz cell was employed (low volume). All samples were measured at room temperature (23 °C). There was insufficient material to obtain optical rotation data for **4**.

3.4. Insects and bioassays

C. maculatus (Fabricius, 1775) originally collected in Ghana (Kestenholtz et al., 2007) were maintained in 5 l glass culture jars with perforated lids in an unlit growth cabinet (28 °C, 55% RH) to develop on cowpea seeds *V. unguiculata*. Under these conditions, adults emerged from cowpea seeds 24–28 days after oviposition. The insects used for bioassays were 3–5 days post-emergence.

Samples of dried extract were re-dissolved in aliquots (10 mL) of solvent to concentrations that represented the proportions in a 10% extract of plant material. **1** (8.1 mg) and **5** (12.1 mg) were dissolved in CH₃CN to 1 mg mL⁻¹ (1000 ppm) and further diluted to 100 and 10 ppm. The lowest concentration was chosen as it approximated the amounts of **1** (5.2 ppm) and **5** (8.1 ppm) in the

Table 4
Analysis of variance (ANOVA) of the mean percent mortality and number of eggs laid per female when bruchids were exposed for six days to crude extracts, isolated *nor*-hopane compounds from *Zanha africana* root bark and compared with a rotenone positive control.

Treatment and concentration	Mean percent mortality	Mean total number of eggs per female
1 10 ppm	49.81 ^{b,c}	11.99 ^b
1 100 ppm	57.69 ^b	13.92 ^b
1 1000 ppm	87.91 ^a	7.97 ^b
5 10 ppm	52.73 ^{b,c}	12.72 ^b
5 100 ppm	55.17 ^{b,c}	9.16 ^b
5 1000 ppm	87.56 ^a	9.47 ^b
Rotenone 10 ppm	94.6 ^a	9.50 ^b
Rotenone 100 ppm	94.40 ^a	5.25 ^b
Rotenone 1000 ppm	97.22 ^a	5.29 ^b
Chloroform 10% w/v	57.54 ^b	10.92 ^b
Methanol 10% w/v	50.09 ^{b,c}	13.55 ^b
Water 10% w/v	57.09 ^b	8.82 ^b
solvent control	27.55 ^c	23.08 ^a

Values in the same column followed by the same letter are not significantly different from each other at the 95% confidence interval using Tukey's post-hoc Honestly Significant Difference (HSD) test.

10% w/v chloroform extract. Aliquots (75 μL) of compounds or extracts were evaporated onto vials (25 mL, nominal capacity) under a stream of air and with constant rotation of the vial. Insects ($N = 5$ –9) were added to the vials, ensuring a ratio of at least 1:1 (male to female). Replicates ($N = 25$) were prepared in this way and the negative control group, which consisted of vials from which 75 μL aliquots each of H₂O, MeOH and CHCl₃ has been sequentially evaporated ($N = 23$). Rotenone, 1000, 100 or 10 ppm, in MeOH, was used as a positive control ($N = 10$, per concentration). After 24, 48 and 72 h mortality was assessed, and cowpeas ($N = 5$) were added to each vial. After a further 72 h, mortality was recorded once more. The numbers of eggs laid on both the vials and the beans were counted and from these data the eggs laid per female were calculated.

3.5. 3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene (1)

Off white solid; UV (MeOH) λ_{\max} nm: 253; $\Delta\epsilon_{336} + 45.7^\circ$ (c 0.54, MeOH); for ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS m/z : 561.3592 [M–H] $^-$ (calc. for C₃₆H₄₉O₅, 561.7713).

3.6. 3 β ,6 β ,11 α -trihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene (2)

Off white solid; UV (MeOH) λ_{\max} nm: 258; $\Delta\epsilon_{336} + 30.6$ (c 0.54, MeOH); for ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS m/z : 577.3541 [M–H] $^-$ (calc. for C₃₆H₄₉O₆, 577.7707).

3.7. 11 α -acetoxy-3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene (3)

Off white solid; UV (MeOH) λ_{\max} nm: 258; $\Delta\epsilon_{336} - 64.00$ (c 0.54, MeOH); for ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS m/z : 619.3653 [M–H] $^-$ (calc. for C₃₈H₅₁O₇, 619.8073).

3.8. 3 β ,6 β -dihydroxy-7 β ,11 α -di[(4-hydroxybenzoyl)oxy]-21 α H-24-norhopa-4(23),22(29)-diene (4)

Off white solid; UV (MeOH) λ_{\max} nm: 258; for ^1H NMR, for ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 1 and 2; HRESIMS m/z : 697.3673 [M–H] $^-$ (calc. for C₄₃H₅₃O₈, 697.8761). Insufficient compound was available to measure optical rotation.

3.9. 3 β ,6 β -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene (5)

Off white solid; UV (MeOH) λ_{\max} nm: 256; $\Delta\epsilon_{336} + 21.1$ (c 0.54, MeOH); for ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 2 and 3; HRESIMS m/z : 561.3588 [M–H] $^-$ (calc. for C₃₆H₄₉O₅, 561.7713).

3.10. 3 β ,6 β ,11 α -trihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-24-norhopa-4(23),17(21)-diene (6)

Off white solid; UV (MeOH) λ_{\max} nm: 258; $\Delta\epsilon_{336} + 16.9$ (c 0.54, MeOH); for ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 2 and 3; HRESIMS m/z : 577.3541 [M–H] $^-$ (calc. for C₃₆H₄₉O₆, 577.7707).

3.11. 6 β ,11 α -dihydroxy-7 β -[(4-hydroxybenzoyl)oxy]-3-oxo-24-norhopa-4(23),17(21)-diene (7)

Off white solid; UV (MeOH) λ_{\max} nm: 255; $\Delta\epsilon_{336} + 13.8$ (c 0.54, MeOH); for ^1H NMR and ^{13}C NMR spectroscopic data, see Tables 2 and 3; HRESIMS m/z : 575.3386 [M–H] $^-$ (calc. for C₃₈H₄₇O₆, 575.7548).

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phytochem.2016.01.008>.

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