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NEW ANTITRYPANOSOMAL TETRANOTRITERPENOIDS FROM *AZADIRACHTA INDICA*

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Abstract

Organic extracts of the leaves of *Azadirachta indica* A. Juss. yielded ten antitrypanosomal terpenoids. Three of these **(1 – 3)**, are novel and are derivatives of nimbolide and nimbin. They were extracted from chloroform fraction of methanol extract. These compounds were found to exhibit strong antitrypanosomal activities against *Trypanosoma brucei rhodesiense* with MIC values ranging of 6.9, 15.6 and 7.8 µg/ml respectively and were more active than Cymerlarsan (a standard drug), which had an MIC value of 187.5 µg/ml when tested against *T. b. rhodesiense* The structures were elucidated by spectroscopic methods including; NMR, MS, UV and IR.

Key words*:* Meliaceae, limonoids, *Trypanosoma brucei rhodesiense, Azadirachta indica,* antitrypanosomal activity.

Introduction

Azadirachta indica (Meliaceae) is a large tree growing in tropical/subtropical regions. The leaves, bark, wood, roots and fruits are intensely bitter. Extracts from different parts of *A. indica* have shown activity against several diseases and their vectors that affect human and animals (Nagui, 1987; Mackinnon et al., 1997). According to Ayurveda (Hindu System of Medicine) the different parts of this tree possess different medicinal properties. A tea prepared from the leaves and bark is used to treat fever (Kokwaro, 1993); a decoction of fresh leaves is used as a favorite wash to cure old ulcers of long standing and the aqueous extract of the leaves in particular is used as remedy for malaria, similar to the practice in Nigeria. The methanol extract of *A. indica* exerts a pronounced anti-inflammatory (rat paw oedema) and a fairly good antipyretic effect (pyrogen induced hyperpyrexia) in rabbits, and thus may justify its use in the treatment of fever resulting from malaria (Okpanyi and Ezeukwu, 1981). The biological activities of *A. indica* have been widely attributed to the presence of limonoids; modified triterpenes with or derived from a precursor with a 4, 4,8-trimethyl-17-furanylsteroid skeleton. Crude extracts of some of the limonoids constituents of the *A. indica* have been shown to exhibit antimalarial activities against chloroquine resistant K1 strain of *Plasmodium falciparum* (Bray et al., 1990). We now report the structures of three new limonoids that were isolated from the leaves of *A. indica* and their activities against *T. b. rhodesiense.*

Materials and Methods

Plant materials

The leaves of A. indica were collected from Shimba Hills National Park, Kwale, South coast of Kenya and a voucher specimen No MG/1/2002 has been deposited in the herbarium of the Botany department of University of Nairobi (Kenya).

Extraction and isolation of compounds from *A. indica*

Air dried and grounded powder of *A. Indica* leaves (6 Kg), was soaked in methanol (10 liters) for 48 hours and then filtered. . The residue was soaked for another 48 hours in methanol (10liters) and then filtered. The combined filtrates were concentrated to dryness under reduced pressure at 45^{o}C to yield a viscous oil (636 g). The extract was partitioned between water (100 ml) and chloroform (250 ml) x 3. The combined organic layer was evaporated to dryness under reduced pressure. Thirty grams of the chloroform layer was column chromatographed on silica gel (column size, 81 x 4.5 cm; 230 - 400 mesh) using hexane/ethyl acetate gradient (90 - 10% hexane). For each solvent system, 1 liter of solvent was used to elute and 50ml fractions collected. Separation was monitored using thin layer chromatography (TLC). The plates were developed in hexane: ethylacetate (2:1), then sprayed with Ehrlich's reagent (2% 4- dimethylaminobenzaldehyde) and developed in hydrogen chloride gas chamber. Typical purplish spots were obtained for limonoids rich fractions, which eluted at 55 - 100% ethylacetate (Maier and Edward, 1970).

A limonoid-bearing fraction (12 mg) was analyzed by semi-preparative HPLC on Beckmann Ultrasphere ODS reverse phase column (250 x 10 mm i.d) on Varian 5000 liquid chromatograph using 1:1 water/acetonitrile isocratic system at a flow rate of 2.5 ml per min and detected at 215 nm. Three new limonoids **1 (**6.9 mg), **2** (15.6 mg) and **3** (7.4 mg), were obtained and tested for antitrypanosomal activity. NMR experiments on the compounds were recorded on Brucker Avance (500 MHz) Spectrophotometer. The ^{1}H and ^{13}C spectra were recorded in $CDCl_3$ solvent. Chemical shifts (δ) were reported in parts per million (ppm) relative to tetramethylsilane (TMS) as the internal standard and coupling constants (J), in hertz (Hz). Peaks on ^{1}H NMR are reported as singlets (s), doublets (d), doublet of doublet (dd), triplets (t) and multlplets (m). The ^{13}C multiplicity was determined by DEPT experiments, which gave chemical shifts values in ppm for assignments.

The IR spectra of the compounds were recorded on a Fourier Transform infrared spectrometer (FT-IR-8101, DR- 8001). Electron Impact Mass Spectra (EIMS) were obtained on a fission VG Platform II GC/LC-MS spectrometer at 70 eV by solid probe. Melting points of the compounds were determined on Sanyo Gallenkamp electronic melting point apparatus (Cal No. MPD350. BM 3.5 Serial No. SG97/12/425 UK and are uncorrected.

Propagation of typanosomes cultures

Trypanosomiasis brucei rhodesiense (KETRI 3438) procyclic forms were obtained from Kenya Trypanosomiasis Research Institute (KETRI) trypanosomiasis bank and stored in liquid nitrogen. They were resuscitated and cultured until correct parastaemia for *in vitro* screening was obtained. The strain was isolated on 7th July 1997 from a human patient and passaged through mice. The pre-patent period was six days while the pre-freezing period was twenty three days.

Antitrypanosomal bioassay

The procedure developed by Burri et al. (1993) to determine the levels of melarsoprol in serum samples of animals after drug treatment was employed. The bioassay was carried out in 96 flat-bottomed well plates with a lid (Corning Glass Works, Corning, New York) arranged in a matrix of 8 rows A - H and 12 columns 1 - 12 (Figure 1). The tests were carried out in columns 11 - 2 and rows B - G. The wells in columns 1 and 12 and rows A and H were not used due to high rate of evaporation. The stock solutions were prepared in sterilized distilled water (1 mg/ml) and diluted to appropriate concentrations. For every extract, three different concentrations were prepared, that is, 500, 250 and 125 µg/ml. Trypanosomes in logarithmic growth phase below 10^6/ml one day after sub-passaging were diluted to 2 x 10^3 cells/ml in medium. Hundred µl of this trypanosome suspension was placed into each well. 100 µl extract solution of each concentration was added to two adjacent wells of column 11. Serial dilutions were carried out by transferring 100 µl from wells of column 11 to wells of column 10 by multi-channel pipettes, then from wells of column 10 to 9, from 9 to 8 and so on up to wells of column 4. For every test, the highest concentration was in column 11 and in twofold dilution to the lowest in column 4 in 128 fold range, so that, for every extract, the highest

concentration was 250 µg/ml and the lowest was 0.488 µg/ml. Columns 3 and 2 were the control, that is, no extract was added. Plates were incubated for 72 hrs at 27°C in 5% CO_2.

Determination of minimum inhibition concentration

The test was observed under inverted microscope at 100-fold magnification. In every row the highest dilution of either standard or samples with less than 5 motile trypanosomes of normal shape was determined. The concentration in this well is defined as the minimum inhibitory concentration (MIC).

$MIC*D*2^n = C_X$

MIC = Minimum Inhibitory Concentration

D = Dilution of extract by medium in wells of column 11 (maximum concentration)

n = Steps of dilution to the first well with no living trypanosomes

Cx = Concentration of extract solution

* = Multiplication

Results and Discussion

The HREIMS of compound **1**gave a molecular ion at m/z 554.2254 corresponding to $C_{31}H_{38}O_9$. ^1H and ^{13}C NMR data (Tables 1 and 2) showed resonances for four distinctive tertiary methyl groups δ_H 1.23 (H-19), 1.68 (H-18), 1.58 (H-29), 1.28 (H-30) on carbons at δ_C 16.4 (q), δ_C 12.8 (q), δ_C 17.1 (q), δ_C 17.5 (q), respectively. The positions of these methyl groups were assigned by analysis of HMBC spectrum. Correlations of H-18 with the carbon resonance at δ_C 134.9 (C-13) and δ_C 146.3 (C-14); H-19 with the carbon resonance at δ_C 202.6(C-1), δ_C 43.6 (C-5), δ_C 41.4(C-9) and δ_C 47.3(C-10); H-29 with the carbon resonance at δ_C 148.5(C-3), δ_C 47.7(C-4), δ_C 43.6(C-5) and δ_C 175.9(C-28) and H-30 with the carbon resonance at δ_C 86.9(C-7), δ_C 49.6(C-8) and δ_C 146.3(C-14) (Figure 2). ^1H NMR spectrum indicated presence of a β-substituted furan moiety at δ_H 7.23, 6.29 and 7.28. Examination of COSY spectra revealed that methine protons at δ_H 6.29 coupled with methine protons at δ_H 7.28, which allowed the assignment of the hydrogen atoms at δ_H 6.29 to C-22, 7.28 to C-23 and 7.23 to C-21. The carbonyl group on C-1 was deduced from the absence of H-1 resonance and was confirmed from HMBC correlation of H-2 δ_H 7.08 (d, J = 9.7Hz), H-3 δ_H 6.44 (d, J = 10.1Hz), H-5 δ_H 3.43(d, J = 11.6Hz), H-9 δ_H 2.67 (t, J = 11.0, 5.5Hz) and methyl protons at C-19 δ_H 1.23(s) with the carbon resonance at δ_C 202.6. Cross peaks between methyl protons at δ_H 1.58(H-29), to carbons at δ_C 148.5(C-3), δ_C 47.7(C-4), δC 43.6(C-5) and δ_C 175.9(C-28) confirmed the position of H-29 and also confirmed C-28 as a ketone. The position of methoxy groups was deduced from HMBC correlations of the methoxy protons on C-12 with the carbon resonance at δ_C 51.6 (OCH_3-12) and of the methoxy protons on C-15 with the carbon resonance at δ_C 50.6 (OCH_3-15) . The structure is proposed to be 7α-acetyl-15β-methoxy-29 methylene 7,15-deoxo nimbolide.

HREIMS of compound **2** gave a molecular ion at m/z 556.1342 corresponding to $C_{31}H_{40}O_9$. IR spectrum indicated the presence of a hydroxyl (3446 cm^{-1}), CH aliphatic stretch (2997 cm^{-1}), C=O (1729 cm^{-1}), olefinic double bond (1652 cm^{-1}) and C-O (1232-1150 cm^{-1}). NMR spectral data showed a basic structure similar to that of nimbolide (**4**) a known compound isolated from ethanoic extract of *A. indica* (Sudaratana et al., 1985). ^1H and ^{13}C NMR spectral data (Tables 1 and 2), showed presence of six tertiary methyl groups δ_H 0.925 (s, H-19), 1.64 (s, H-18), 1.12 (s, H-29), 1.28 (s, H-30), 2.17 (s, H-1a) and 1.95 (s, H-1b). The positions of these methyl groups were assigned by analysis of HMQC and HMBC correlations (Figure 2). Examination of COSY spectrum revealed that methine protons at δ_H 2.58 (dd, J = 9.5, 7.1, 2.4 Hz, 1H) coupled with methylene protons at δ_H 2.03 (d, J = 16.1Hz, 2H). HMBC correlation of the proton at δ_H 2.03 with carbons at resonance δ_C 172.5 (carbonyl carbon) and 39.3 allowed the assignment of protons at δ_H 2.03 to C-11 and 2.58 to C-9. From HMBC correlations, cross peaks between methyl protons at δ_H 1.95 (s), δ_H 2.17 (s) with carbons at δ_C 158.3 and δ_C 115.4 and cross peaks between protons at δ_H 5.74 (s) with carbons at δ_C 164.7, 20.4 and 27.5 suggests the side chain to be tigloyl. HMBC correlation of methine proton at δ_H 3.61 (t, 7.2Hz, 1H) with furan carbons allowed the assignment of that proton to C-17 (δ_C 49.3). The HMBC cross peaks between methyl protons at δ_H (1.64, s, 3H) with carbons at δ_C 134.7, 146.7 and 49.3 permitted the assignment of quartenary carbons C-13 and C-14. There is a double bond between them as they have no HMQC correlation and no COSY coupling. From ^1H and ^{13}C NMR there is one methoxy group whose position was revealed by the HMBC correlation. The assignment of relative stereochemistry was based on the NOESY experiments. The H-9α resonance showed a NOESY correlation with OH-3, H-5, H-15 and H-16α confirming the α orientation of H's on C-5, C-15 and hydroxyl group on C-3. H-16β resonance showed a NOESY correlation with 3H-30β, H-7, 3H-19β and 3H-29β whereas H-17 resonance showed a NOESY correlation with 3H-

19β, 3H-29β and H-16β. NOESY correlation of H-6 with H-7, H-3, 3H-29β and 3H-30β confirmed the β-orientation of H's on C-3, C-6, C-7 and C-17 (Figure 3). The compound is proposed to be 2-oxo-3-deacetyl salannin.

Compound **3** has a molecular formula $C_{30}H_{38}O_{10}$ (m/z 558). The IR spectrum indicated presence of a hydroxyl group (3427 cm^{-1}), CH aliphatic stretch (2924 cm^{-1}), C=O (1732 cm^{-1}), olefinic double bonds (1680 cm^{-1}) and C-O (1234 cm^{-1}). ^{1}H and ^{13}C NMR spectral data (Tables 1 and 2 indicated the presence of a β−substituted furan moiety at δ_H 7.2 (H-21, s), 6.32 (H-22, s) and 7.31 (H-23, d, 9.9 Hz) corresponding to carbons at δ_C 138.9 (d), 110.3 (d) and 143.0 (d), respectively. The signals at δ_C 148.0 (d), 126.7 (d) and 202.1 (s) corresponding to protons at δ_H 6.41 (1H, d, J = 4.1 Hz) and 5.8 (1H, dd, J = 16.6, 9.7, 6.9 Hz) are typical of α, β- unsaturated ketone moiety in the A-ring. HMBC correlation of protons at δ_H (5.86, dd, J = 16.6, 9.7, 6.9 Hz), 6.41 (d, J =4.1 Hz), 3.39 (d, J = 12.6 Hz), 2.74, (t, J = 9.3, 3.8 Hz) and methyl protons at 1.20(s) with the carbon resonance at δ_C 202.1(s) allowed the assignment of δ_H 6.41 to C-3 and proton at δ_H 5.86 to C-2 and carbonyl to C-1. This was confirmed by the examination of COSY spectra which revealed that methine protons at δ_H 6.41 (1H, d, J = 4.1 Hz) coupled with methine protons at δ_H 5.86 (1H, dd, J = 16.6, 9.7, 6.9 Hz). ^{1}H and ^{13}C NMR indicate the presence of 2 methoxy groups attached to keto carbons. The compound is proposed to be 7α-hydroxy-15β-hydroxy-7,15-deoxo nimbin

Table 1: ^{1}H NMR spectral data for compounds 1-3 [δ_H ppm, (multi, JHz)]

H	**1**	**2**	**3**
1		4.99 (s)	
2	7.08(d, J = 9.7)		5.86 (dd, J = 16.6, 9.7, 6.9)
3	6.44(d, J =10.1)	3.83 (d, J = 9.4)	6.41 (d, J = 4.1)
5	3.43 (d, J = 11.6)	2.64 (d, J = 11.7)	3.39 (d, J = 12.6)
6	3.79 (d, J = 7.1)	3.97 (dd, J = 11.4, 9.1, 3.1)	4.01 (d, J = 3.2)
7	4.01 (d, J = 3.2)	4.15 (d, J = 3.1)	3.88 (m)
9	2.67 (t, J = 11.0, 5.5)	2.58 (dd, J = 9.5, 7.1, 2.4)	2.74 (t, J = 9.3, 3.8)
11	2.33 (dd, J = 16.2	2.03 (d, J = 16.1)	2.22 (d, J = 3.6)
11	3.27 (d, J = 16.1, 5.4, 0.7)	2.26 (m. J = 12.7, 6.4)	2.86 (dd, J =13.5, 6.3, 5.5)
15	5.57 (t, J = 7.8, 6.6)	5.41(t, J = 14.2, 7.2))	5.54 (t, J = 14.4, 6.3)
16	2.22 (m)	2.13 (m)	2.03 (m)
16	2.89 (dd, J = 16.1, 5.4, 0.7)	2.23 (m)	2.18 (m)
17	2.07 (m)	3.61 (t, J = 7.2)	2.81 (d, J = 2.7)
18	1.68 (s)	1.64 (s)	1.67 (s)
19	1.23 (s)	0.92 (s)	1.20 (s)
21	7.23 (s)	7.30 (s)	7.2 (s)
22	6.29 (d, J = 12.2)	6.29 (s)	6.32 (s)
23	7.28(d, J = 9.9)	7.24 (s)	7.31 (d, J = 9.9)
28		4.09 (d, J = 8.3)	
29	1.58 (s)	1.12 (s)	1.58 (s)
30	1.28 (s)	1.28 (s)	1.28 (s)
3OH		5.82 (s)	
6CH_3			1.32 (s)
7CH_3	1.36 (s)		
12OCH_3	3.56 (s)		3.64 (s)
15OCH_3	3.68 (s)		
28a	4.23(s)		
1b		5.74 (s)	
12OCH_3		3.17 (s)	
1a CH_3		2.17 (s)	
1b CH_3		1.95 (s)	
150H			3.79 (s)

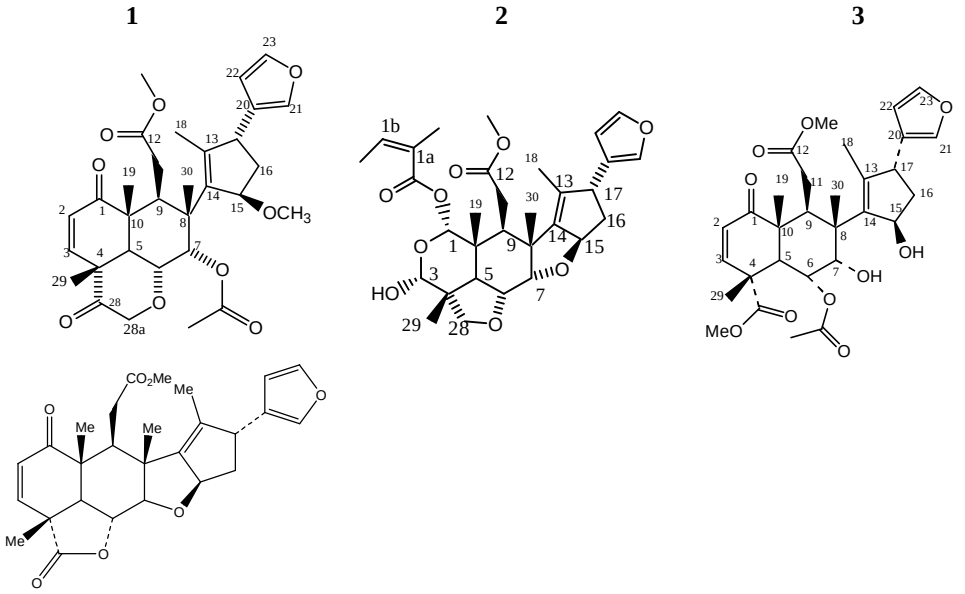

Figure 1: Chemical structures of compounds **1-4**

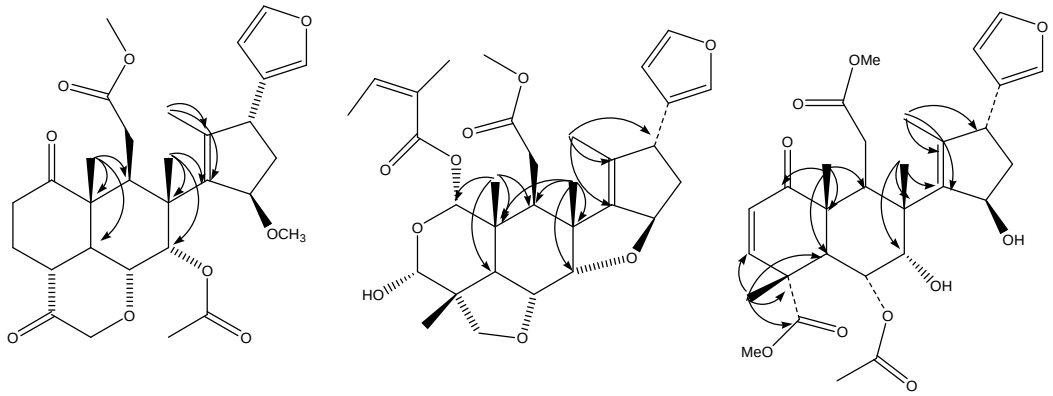

Figure 2: Long range HMBC correlation elucidating methyl groups

Top face Lower face

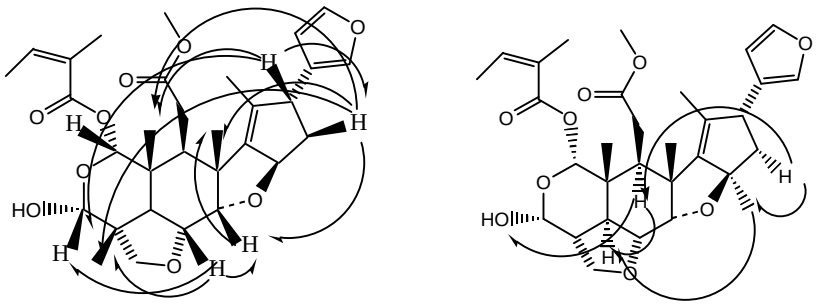

Figure 3: NOESY correlation of compound **2**

Table 2: ^{13}C NMR spectral data for compounds 1-3 (δ_C (ppm)

carbon	**1**	**2**	**3**
1	202.6 (s)	71.8 (d)	202.1 (s)
2	126.4 (d)		126.7(d)
3	148.5 (d)	71.0 (d)	148.0 (d)
4	47.7 (s)	44.2 (s)	47.7 (s)
5	43.6 (d)	38.8 (d)	43.6 (d)
6	66.2 (d)	72.4 (d)	87.3 (d)
7	86.9 (d)	85.7 (d)	66.1 (d)
8	49.6 (s)	48.9 (s)	47.4 (s)
9	41.4 (d)	39.3 (d)	39.0 (d)
10	47.3 (s)	40.4 (s)	47.4 (s)
11	32.5 (t)	39.4 (t)	34.3 (t)
11	32.5 (t)	30.4 (t)	34.3 (t)
12	173.6 (s)	172.5 (s)	173.6 (s)
13	134.9 (s)	134.7 (s)	134.8 (s)
14	146.3 (s)	146.7 (s)	146.7 (s)
15	87.7 (d)	87.7 (d)	86.8 (d)
16	34.2 (t)	41.0 (t)	41.4 (t)
16	34.2 (t)	41.0 (t)	41.4 (t)
17	41.9 (d)	49.3 (d)	51.1(d)
18	12.8 (q)	13.1 (q)	12.5 (s)
19	16.4 (q)	15.3 (q)	16.3 (s)
20	126.8 (s)	127.1 (s)	126.3 (s)
21	138.9 (d)	142.7 (d)	138.9 (d)
22	110.4 (d)	110.7 (d)	110.3 (d)
23	148.1 (d)	138.7 (d)	143.0 (d)
28	175.9 (s)	78.3 (t)	173.6(s)
29	17.1 (q)	19.7 (q)	17.1 (s)
30	17.5 (q)	16.9 (q)	17.5 (s)
7C=O	174.1 (s)		152.9 (s)
$7CH_3$	20.5 (q)		
12C=O	173 (s)		
$12OCH_3$	51.6 (q)	51.3 (q)	51.6 (q)
$15OCH_3$	50.6 (q)		
28a	85.3 (d)		
1C=O		164.7 (s)	
1a		158.3 (s)	
1b		115.4 (d)	
$1aCH_3$		20.4 (q)	
$1bCH_3$		27.5 (q)	
6C=O			152.9 (s)
6Me			20.0 (s)

Antitrypanosomal Activity

Crude methanolic extracts and chloroform fractions of *A. indica* leaves exhibited significant antitrypanosomal *in vitro* activities against *T. b. rhodesiense* procyclic forms (KETRI 3438) whose MIC values are 51.2±0 and 4.4 ± 0 µg/ml, respectively and exhibited higher activities than cymerlarsan (drug in the market) which had an MIC value of 187.5 µg/ml. Chloroform extracts were more potent than the methanol extracts, indicating that the active compounds are of medium polarity.

Repeated chromatographic separations of the chloroform fraction yielded three new tetranotriterpenoids (**1 - 3**) that were tested against *T. b. rhodesiense* procyclic forms (KETRI 3438). The MIC values were 6.9 ± 0, 15.6 ± 0 and 7.8 ± 0 µg/ml, respectively. All the three compounds had lower activities than that of the chloroform fraction suggesting that there are other antitrypanosomal compounds present or possible synergistic blend effects. Based on

their spectroscopic data and comparison with published data, the structures of the compounds were readily elucidated and were found to have the nimbolide (**4**) basic structure (C-seco limonoids). Compound **2** which has an epoxy group between C_7 and C_{15} has the lowest activity whereas compounds **1** and **3** with no epoxy group and with more methoxy groups exhibited higher activities. This suggests that antitrypanosomal activity can be enhanced by chemical modifications of the basic structure (nimbolide) and its derivatives to generate new effective antitrypanocidal drugs with novel modes of action.

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