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ORIGINAL ARTICLE

In vitro anthelmintic and cytotoxic activities of extracts from the stem barks of *Berlinia confusa* (C. Hoyle) and identification of its active constituents

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KEYWORDS

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Abstract Phytochemical investigation of EtOAc extract from the stem bark of *Berlinia confusa* yielded a new and two known polysaturated monoacylglycerides characterised as 1-*O*-docosanoyl-sn-glyceride (**3**), 1-*O*-(13-methyltetradecanoyl)-sn-glycerol (**4**) and 1-*O*-pentadecacanoyl-sn-glycerol (**5**); along with the known compounds betulinic acid (**1**) and sitosteryl-β-D-glucoside (**2**). The structures of these compounds were elucidated using analytical methods, including 1D and 2D-NMR together with MS spectroscopy. The extracts and isolated compounds demonstrated concentration-dependent anthelmintic activities against *Fusciola gigantica* (liver flukes) and *Taenia solium* (tapeworm) at 10–100 mg/ml. The extracts and isolated compounds were evaluated for cytotoxicity against a small panel of three human tumour cell lines.

1. Introduction

Berlinia confusa (C. Hoyle), Leguminoseae (Fabaceae) is a woody perennial tree widely distributed throughout the high rainforest and semi-deciduous forest zones of West Africa, stretching from Sierra-Leone to Nigeria through Cameroun to Garbon (Voorhoeve, 1965; Savil and Fox, 1967; Keay, 1989). *B. confusa* has many Nigerian vernacular names such as *abaila* (Yoruba), *ekpogoi* (Edo), *idok* (Igala), *ayera* (Esan) and *ogba* (Igbo). The stem-barks infusion of *B. confusa* is used in South-Western Nigeria for the treatment of gastro-intestinal worms (Irvin, 1961). In the Democratic Republic of Congo, Ghana, Liberia and Sierra-Leone, leaves decoction of *B. confusa* is used as ingredient of ceremonial sauces, tonic and herbs for hastening child birth (Voorhoeve, 1965). Previous study on the chemical constituents and pharmacology of *Berlinia grandiflora* revealed the presence of betulinic acid, an anthelmintic compound (Enwerem et al., 2001). In developing countries like Nigeria, helminth infections are a major health concern because they predispose humans to other infections such as

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bacterial and fungal infections (Cox, 2001). Such helminth infections can lead to serious diseases among poor people due to poor sanitation, poverty and malnutrition (Brooker et al., 2006). Worm infections have been treated locally in Nigeria. Among the plants used by the Nigeria native includes *B. confusa* and *B. grandiflora* (Voorhoeve, 1965; Ajaiyeoba and Okogun, 1994; Asuzu et al., 1993).

In a preliminary screening of plants used for gastro-intestinal worm infections, EtOAc soluble portions of the methanolic extract from *B. confusa* displayed significant anthelmintic activity against *Fusciola gigantica* (liver flukes) and *Taenia solium* (tapeworm). This result motivated phytochemical investigation in order to isolate active compounds responsible for the anthelmintic property. Bioassay-guided separation of the EtOAc extract using column chromatography afforded fractions consisting primarily of polysaturated fatty acid-containing monoglyceride. One new and two known monoacyl glycerides were successively isolated and characterised as 1-*O*-docosanoyl-sn-glyceride (**3**), 1-*O*-(13-methyltetradecanoyl)-sn-glycerol (**4**) and 1-*O*-pentadecacanoyl-sn-glycerol (**5**), respectively. In addition, 3β-hydroxyl-lup-20(29)-ene-28-oic acid (betulinic acid, **1**) and sitosteryl-β-D-glucoside (**2**) were isolated. Details of the isolation, structural determination, anthelmintic and cytotoxic properties are described herein.

2. Experimental

2.1. General

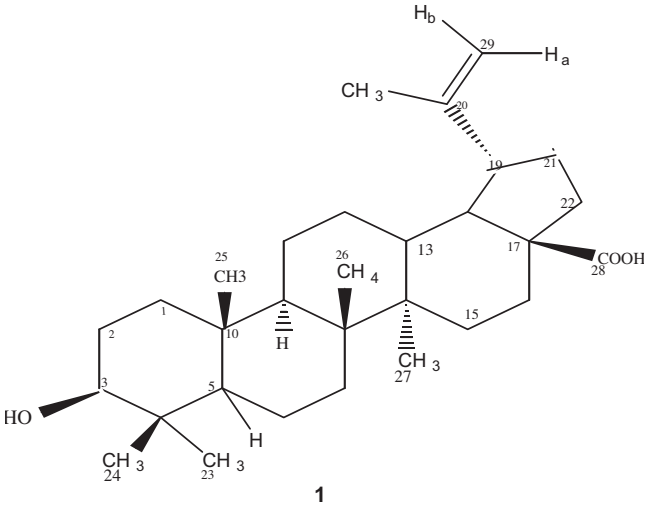
Melting points were determined on Gallenkamp (Phillip Harris, England) apparatus and are uncorrected. Optical rotations are measured on a Perkin–Elmer 341 polarimeter. The analytical and preparative procedure utilised adsorption chromatography. Analytical thin layer chromatography (TLC) was performed using commercial pre-coated plates (Kieselgel, 60 PF_{254}, 0.25 mm thick on polyester backing, Merck, Darmstadt, Germany (Merck Ltd.). Column chromatography was performed on open column silica gel (Merck, Kieselgel 60, 70–230 mesh). ESI-MS data were obtained on a JEOL JMS 700T and the mobile phase composition was methanol. ^{1}H- and ^{13}C-NMR spectra were taken in deuteorochloroform ($CDCl_3$) in the Fourier transform mode on a Bruker WP 120,054 Spectrometer at 400 and 100 MHz (for the ^{1}H- and ^{13}C-NMR), respectively, δ in ppm with TMS as internal reference standard. The coupling constant, *J* values were recorded in Hertz (Hz).

2.2. Plant material

The stem barks of *Berlinia confusa* (C. Hoyle) were collected at the premises of the Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State, Nigeria, by Mr. T.K. Odewo (taxonomist) in October 2005. The plant material was authenticated at FRIN through comparison with the voucher specimen in the herbarium, under the accession number FH1 147825.

2.3. Extraction and isolation

Dried and powdered stem barks of *B. confusa* (C. Hoyle, 3.5 kg) was extracted by percolation with MeOH (48 h, reflux) using a large aspirator bottle fitted with extraction gadgets. The extract was concentrated in vacuo, at reduced pressure to afford MeOH extract (250 g). The resultant methanolic extract was suspended in distilled water (10 L) and successively partitioned in hexane and EtOAc. The extracts were concentrated at reduced pressure, to yield hexane (18 g) and EtOAc extract (7 g). The MeOH and EtOAc extracts from the stem barks of *B. confusa* displayed significant anthelmintic activities on the *Fusciola gigantica* and *Taenia solium* worms, also a moderate toxicity against brine shrimp larvae. The EtOAc soluble portions (7 g) were chromatographed on silica gel (Kieselgel 60, 70–230 mesh) packed column, eluting with gradient of solvents system of diethyl ether (Et_2O): n-hexane (0:1→1:0, 100 ml vol/vol), and EtOAc/Et_2O [(0:1→1:0), 100 ml vol/vol)]. Using TLC analysis, 100 fractions obtained were grouped into eight sub-fractions coded A–H. All fractions were evaluated for *in vitro* anthelmintic and cytotoxic activities. Fraction B eluted with 30% ether in hexane afforded betulinic acid (**1**, 200 mg) after recrystallising in EtOAc/hexane (3:7, vol/vol, 30 ml). Compound **1** displayed remarkable anthelmintic activities on the worms assayed. Fractions C and D were too small to allow further purification. Fraction E eluted with 80% Et_2O in hexane afforded an amorphous white solid, which on further purification in 100% isopropyl alcohol and recrystallisation in EtOAc/hexane solvent mixture (3:7, vol/vol) yielded white powder, sitosteryl-β-D-glucoside (**2**, 500 mg). Compound **2** exhibited moderate cytotoxic and anthelmintic activities. Fraction H eluted with 15% EtOAc in Et_2O yielded white gum. Further purification in isopropyl alcohol and recrystallisation in EtOAc/hexane solvent system (3:7, vol/vol, 200 ml) afforded white powder, 1-*O*-docosanoyl-sn-glyceride (**3**, 450 mg). Fraction F was further rechromatographed in open CC, eluting with Et_2O/EtOAc (0:1→1:0, vol/vol, 50 ml) to afford 10 fractions. Fraction 6 eluted with 30% EtOAc in Et_2O yielded white amorphous solid, recrystallised in 100% isopropyl alcohol and characterised as 1-*O*-(13-methyltetradecanoyl)-sn-glycerol (**4**, 105 mg). Fraction 10 eluted with 50% EtOAc in Et_2O and afforded pure amorphous solid, 1-*O*-pentadecacanoyl-sn-glycerol (**5**, 300 mg).



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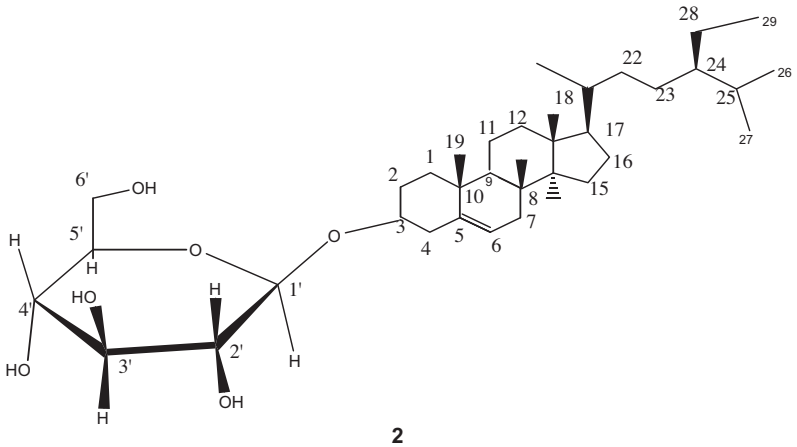

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*2.3.1. 3β-Hydroxyl-lup-20(29)-en-2-oic acid (**1**)*

Compound **1** was isolated as a white powder (200 mg); R_f = 0.60 (benzene/ethyl acetate; 3:1, vol/vol), mp = 208–209 °C (lit. mp = 210 °C); IR: KBr, υ_{max} (cm^{-1})]: 3450 cm^{-1} broad, O–H stretch [H-bonded], 2900 cm^{-1}[C–H_{str}], 1680 cm^{-1} [C═C_{str}], 1711 cm^{-1}[C═O_{str}] and 1450 cm^{-1}; ^{1}H-NMR δ (ppm), (400 MHz, $CDCl_3$): 4.71 [1H dd, *J* = 1.8, 2.7 Hz, H-29a], 4.59 [1H d, *J* = 1.8 Hz, H-29b], 3.02 [1H m, H-3], 1.71 [3H s, H-23], 1.01 [3H s, H-30], 0.98 [3H s, H-25], 0.96 [3H s, H-26], 0.86 [3H s, H-27] and 0.75 [3H s, H-24]; ^{13}C-NMR: 100 MHz, CD_3OD) δ (ppm): 180.2 [C-28], 152.1[C-20], 110.3 [C-29], 79.8 [C-3], 57.04 [C-5], 52.17 [C-17], 50.62 [C-9], 48.64 [C-19], 43.74 [C-18], 42.09 [C-14], 40.24 [C-8], 38.78 [C-1], 38.49 [C-4], 38.28 [C-13], 35.76 [C-10], 33.50 [C-7], 31.87 [C-16], 30.99 [C-15], 29.8 [C-21], 28.76 [C-22], 28.20 [C-23], 27.05 [C-2], 22.40 [C-12], 19.70 [C-11], 19.60 [C-30] 16.80 [C-25], 16.70 [C-26], 16.25 [C-6], and 15.30 [C-27]; HREIMS: *m*/*z* (rel. int. %) 456 [M^+, 5%], 423 (2%), 302 (1%), 259 (3%), 248 (30%), 235 (12%), 221 (17%), 207 (42%), 189 (100%), 174 (33%), 161 (21%), 147 (32%), 135 (53%), 119 (59%), 107 (63%), 81 (38%), 69 (66%), 55 (97%).

*2.3.2. Sitosteryl-β-D-glucoside (**2**)*

Compound **2** was isolated as a white solid (500 mg); mp = 274–276 °C [lit. mp = 273 °C]; TLC: R_f = 0.30 (mobile phase: $CHCl_3$-EtOAC 4:1 vol/vol); ^{1}H-NMR ($CDCl_3$/CD_3OD, 1:1 vol/vol, 400 MHz) δ (ppm): 5.23 [1H m, H-6], 4.27 [1H d, *J* = 7.8 Hz, H-1′], 3.68–3.74 [2H, 1H each dd (3.74 (1H dd, *J* = 11.58, 5.8 Hz); (3.68 (1H dd, *J* = 11.6, 2.9 Hz, H-6′)], 3.45 [1H, m, H-3], 3.38–3.44 (1H m, H-3′], 3.35–3.42 [1H m, H-4′], 3.28 [1H t, *J* = 7.76 Hz, H-21], 3.27–3.30 [1H, m, H-5′], 0.87 [3H, s], 0.79 [3H, s], 0.73 [3H, s], 0.71 [3H, s], 0.68 [3H, s] and 0.55 [3H, s]; ^{13}C-NMR ($CDCl_3$:CD_3OD, 1:1 vol/vol, 100 MHz) δ (ppm): 140.06 [C-5], 121.89 [C-6], 100.87 [C-1′], 78.91 [C-3], 76.1 [C-3′], 75.6 [C-5′], 73.32 [C-2′], 69.7 [C-4′], 61.1 [C-6′], 56.51 [C-14], 55.81 [C-17], 49.95 [C-9], 45.62 [C-24], 42.08 [C-13], 39.52 [C-12], 38.44 [C-4], 37.01 [C-1], 36.47 [C-10], 35.89 [C-20], 33.69 [C-22], 31.67 [C-8], 31.64 [C-7], 29.35 [C-2], 28.90 [C-25], 27.98 [C-16], 25.81 [C-23], 24.02 [C-15], 22.81 [C-28], 20.81 [C-11], 19.46 [C-27], 19.01 [C-19], 18.68 [C-26], 18.46 [C-21], 11.63 [C-29] and 11.54 [C-18].

Table 1 ^{1}H- and ^{13}C-NMR data of compound **1**.

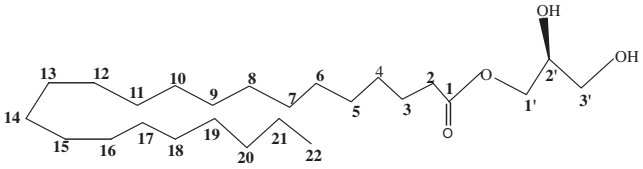
Carbons	δ_H	δ_C	DEPT
1′	4.21, dd (7.02, 4.2) 4.18, dd (5.94, 7.02)	65.2	CH_2
2′	3.94, m	70.2	CH
3′	3.72, dd (8.6, 5.6) 3.62, dd (5.2, 8.6)	63.3	CH_2
1	–	174.4,s	C
2	2.36, t (7.6)	34.2,t	CH_2
3	1.64, tt (7.8, 7.5)	31.9,t	CH_2
4	1.22–1.30, m	29.0,t	CH_2
5	1.22–1.30, m	29.9, t	CH_2
6	1.22–1.30, m	29.8, t	CH_2
7	1.22–1.30, m	29.7, t	CH_2
8	1.22–1.30, m	29.6, t	CH_2
9	1.22–1.30, m	29.5, t	CH_2
10	1.22–1.30, m	29.5, t	CH_2
11	1.22–1.30, m	29.5, t	CH_2
12	1.22–1.30, m	29.4, t	CH_2
13	1.22–1.30, m	29.2, t	CH_2
14	1.22–1.30, m	29.1, t	CH_2
15	1.22–1.30, m	29.1, t	CH_2
16	1.22–1.30, m	29.1, t	CH_2
17	1.22–1.30, m	29.1, t	CH_2
18	1.22–1.30, m	29.1, t	CH_2
19	1.22–1.30, m	29.1, t	CH_2
20	1.22–1.30, m	29.1, t	CH_2
21	1.22–1.30, m	29.1, t	CH_2
22	1.22–1.30, m	24.9, t	CH_2
23	1.22, t	22.7, t	CH_2
24	0.88, t	14.1, t	CH_3

^{1}H- and ^{13}C-NMR spectra data were acquired in $CDCl_3$ at 400 MHz. Chemical shifts values are shown in the δ scale (ppm), with coupling constants (*J*, Hz) in parentheses, s = singlet, d = doublet, t = triplet, m = multiplet.

*2.3.3. 1-O-tetracosaenoyl-sn-glycerol (**3**)*

Compound **3** was isolated as a white powdery solid (450 mg); mp 259–260 °C; TLC: R_f = 0.50 (mobile phase: $CHCl_3$-EtOAC 3:1); ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 4.21

[1H dd, J = 7.02, 4.2 Hz, H-1′], 4.18 [1H dd, J = 5.94, 7.02 Hz, H-1′], 3.94 [1H m, H-2′], 3.72 [1H dd, J = 8.6, 5.6 Hz, H-3′], 3.62 [1H dd, J = 5.2, 8.6 Hz, H-3′], 2.36 [2H t, J = 7.6 Hz, H-2], 1.64 [2H tt, J = 7.8, 7.5 Hz, H-3], 1.22–1.30 [m, 2H each, H-4–H-22], 0.88 [3H t, J = 6.6 Hz, H-24] (Table 1); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 174.35, s [C-1], 70.2, d [C-2′], 65.2 [C-1′], 63.3, d [C-3′], 34.2, t [C-2], 31.9 t [C-3], 29.0–29.88, t [C-4–C-21], 24.9, t [C-22], 22.7, t [C-23], 14.11 [C-24] (Table 2); HR-ESI-MS $[M + NH_4]^+$ ion (m/z 460.3120).



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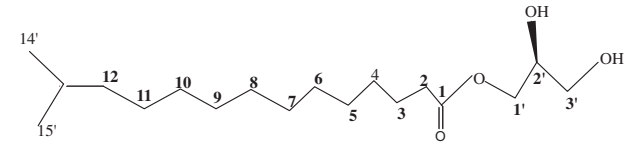
2.3.4. 1-O-(13-methyltetradecanoyl)-sn-glycerol (*4*)

Compound **4** was isolated as a white amorphous powdery solid (105 mg); mp = 245 °C [lit. mp = 243 °C]; $[\alpha]_D^{21}$ + 3.4° (c 0.10, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm) 4.02, (1H, dd, J = 10.6, 4.3 Hz, H-1$_a$), 4.01 (1H, dd, J = 10.82, 6.35 Hz, H-1$_b$), 3.79 (1H, quintet, J = 4.46 Hz, H-2), 3.56 (2H, m, H-3), 2.31 (2H, t, J = 6.95 Hz, H-2′), 1.61 (2H, m, H-3′), 1.51 (1H, m, H-13′), 1.20–1.32 (16H, m, H-4′–11′), 1.16 (2H, m, H-12′), 0.87 (6H, d, J = 6.48 Hz, H-15′); ESI-MS m/z 339 $[M + NH_4]^+$, (100), 317 $[M + H]^+$ (29).



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2.3.5. 1-O-pentadecacanoyl-sn-glycerol (*5*)

Compound **5** was isolated as a white amorphous powdery solid (300 mg); mp = 252 °C [lit. mp = 251 °C]; $[\alpha]_D^{21}$ + 2.8° (c 0.11, MeOH); ^{1}H NMR (CD_3OD, 500 MHz) δ (ppm) 4.12, (1H, dd, J = 10.6, 4.4 Hz, H-1$_a$), 4.01 (1H, dd, J = 10.82, 6.35 Hz, H-1$_b$), 3.79 (1H m, H-2), 3.56 (2H m, H-3), 2.31 (2H t, J = 6.95 Hz, H-2′), 1.61 (2H, m H-3′), 1.20–1.32 (20H, m, H-4′–13′), 1.16 (2H, m, H-14′), 0.87(3H, t, J = 6.48 Hz, H-15′); ESI-MS m/z 339 $[M + NH_4]^+$, (100), 317 $[M + H]^+$ (48).

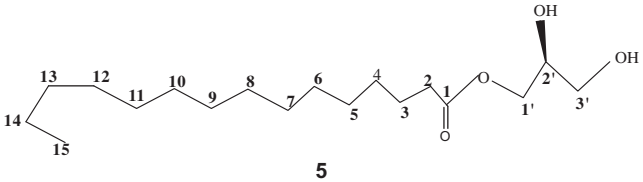

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Table 2 Anthelmintic activity of extracts from *B. confusa*.

Extracts	Conc. (mg/mL)	*Fusciola gigantica*		*Taenia solium*	
		P	D	P	D
MeOH extract	10	50.10 ± 0.1	> 70	20 ± 0.1	35 ± 0.5
	20	40.2 ± 0.3	60 ± 0.2	18 ± 0.3	30 ± 0.2
	50	16 ± 0.2	50 ± 0.7	14 ± 0.5	25 ± 0.1
	100	10 ± 0.3	40 ± 0.5	10 ± 0.2	10 ± 0.1
EtOAc extract	10	34 ± 0.3	22 ± 0.3	16 ± 0.3	25 ± 0.1
	20	30 ± 0.2	22 ± 0.3	14 ± 0.2	22 ± 0.2
	50	24 ± 0.1	19 ± 0.2	14 ± 0.2	22 ± 0.2
	100	20 ± 0.3	18 ± 0.3	12 ± 0.1	20 ± 0.4
Distilled water	–	–	–	–	–

P = mean paralysis time in min, D = mean death time in min.

Table 3 Anthelmintic activities of compounds **1–5**.

Compounds	Conc. (mg/mL)	*Fasciola gigantica*		*Taenia solium*	
		P	D	P	D
1	10	40 ± 0.1	80 ± 00.3	130 ± 0.2	180 ± 0.2
	50	30 ± 0.2	50 ± 0.7	120 ± 0.4	170 ± 0.1
2	10	38 ± 0.3	70 ± 0.5	110 ± 0.3	160 ± 0.3
	50	26 ± 0.2	55 ± 0.1	105 ± 0.2	158 ± 00.2
3	10	32 ± 0.3	60 ± 0.3	100 ± 0.1	150 ± 0.4
	50	24 ± 0.2	40 ± 0.3	92 ± 0.2	140 ± 0.6
4	10	24 ± 0.4	50 ± 0.2	95 ± 0.1	130 ± 0.2
	50	30 ± 0.2	35 ± 0.1	90 ± 0.1	110 ± 0.4
5	10	20 ± 0.1	40 ± 0.3	85 ± 0.2	100 ± 0.3
	50	26 ± 0.2	30 ± 0.2	70 ± 0.3	90 ± 0.2
Piperazine citrate	10	20 ± 0.3	60 ± 0.3	15 ± 0.05	120 ± 0.1
Distilled water	–	–	–	–	–

P = mean paralysis time in min, D = mean death time in min.

2.4. Animal material

Fusciola gigantica (liver fluke, mean weight of 0.06–0.08 g) and *Taenia solium* (tape worm, mean weight 2.5–2.9 g) were obtained from freshly slaughtered cows at *Alogi* abattoir in Abeokuta, Ogun State, Nigeria. The worms were authenticated at the Parasitological Research Unit, Zoology Department, University of Agriculture, Abeokuta, Ogun State, Nigeria.

2.5. Anthelmintic assay

Chemicals used for anthelmintic assay were of IP/BP specifications. Liver fluke and tapeworms of the same type and size were used for evaluating anthelmintic activity. Piperazine citrate was used as standard anthelmintic drug. The method employed was as described previously (Asuzu et al., 1999) with slight modifications. Five worms (of the same types) were placed in 9 cm petri dishes containing 10, 20, 50, 100 mg/mL solution of crude extracts. Five worms of approximately the same size were placed in each petri dish containing 50 mL solution of each extract. Isolated compounds and standard drug were treated in the same way. This was done in duplicate for

Table 4 Cytotoxic activity of isolated compounds from *B. confusa*.

Compounds	IC_{50} (μM)		
	Hela	HL-60	A-549
1	18.1	14.2	150
2	14.1	17.6	160
3	35.2	20.8	180
4	34.1	22.1	170
5	33.7	22.5	175
Adrimycin*	0.20	1.25	5.81

Adrimycin* was used as positive control.

all worms. A control test having five worms in 50 mL distilled water was conducted simultaneously. The mean time (min) required for paralysis was recorded when no movement of any sort was observed, except when the worm was shaken vigorously or transferred into a beaker containing hot water at 50 °C. The death time was recorded after ascertaining the worms neither move when shaken vigorously nor when dipped in hot water (70 °C). The anthelmintic results of the extracts and isolated compounds are presented in Tables 3 and 4.

2.6. Cytotoxicity assay

The cancer cell lines were maintained in RPMI 1640, which included 1-glutamic acid with 10% FBS and 2% penicillin–streptomycin. Cells were cultured at 37 °C in a 50% CO_2 incubator. Cytotoxicity activity was determined following a modified MTT assay (Lin and Tome, 1991; Ajaiyeoba et al., 1998). Viable cells were seeded in the growth medium (120 mL) into 96-well microlitre plates (1×10^4 cells per well) and incubated at 37 °C in a 50% CO_2 incubator. The test sample was dissolved in DMSO and adjusted to final sample concentrations ranging from 5.0 to 150 μM by diluting with the growth medium. Each sample was prepared in triplicate. The final DMSO concentration was adjusted to $< 0.1\%$. After standing for 24 h, 10 μL of the test sample was added to the control wells. On removing the medium after 48 h of the test sample treatment, MTT (5 mg/ml, 10 μL) was equally added to each well. After 4 h incubation, the plates were removed, and the resulting formazan crystals were dissolved in DMSO (150 μL). The OD was measured at 570 nm. The IC_{50} value was defined as the concentration of sample that reduced absorbance by 50% relative to the vehicle-treated control.

2.7. Statistical analysis

Data are presented as the mean ± SEM from four separated experiments. Statistical analyses were performed using the Bonferroni *t*-test method after ANOVA for multigroup comparison and the Student's *t*-test method for two-group comparison. $P < 0.05$ was considered significant. Analysis of linear regression (at least three data within 20–80% inhibition) was used to calculate IC_{50} values.

3. Results and discussion

EtOAc extract of *B. confusa* was subjected to silica gel CC to afford compound **3**. Compound **3** was obtained as white powder; mp 259 °C; optical rotation $[\alpha]_D^{25}$ + 4.34° (c 0.12, MeOH); UV (λ_{max}), no maximum; IR (KBr pellet) are at 3440, 2900 and 1740 cm^{-1}, suggestive of O–H, C–H and C=O stretches, respectively. High resolution electron spray ionisation mass spectrometry (HR-ESI-MS) of compound **3** revealed ammoniated molecular ion peak $[M + NH_4]^+$ at *m/z* 432.201, consistent with the molecular formula $C_{25}H_{50}O_4NH_4$. ^{1}H- and ^{13}C-NMR spectral of compound **3** featured a characteristic pattern of monoacyl glyceride skeleton. The ^{1}H-NMR spectrum exhibited a signal for triplet peak at δ 0.88 (3H, J = 6.6 Hz, H-25) ascribable to methyl protons. The multiplets between δ 1.22 and 1.30 (2H each, H-4–20) were assigned to methylene protons in the suggested structure of **3**. A triplet peak occur at δ 2.36 (2H t, J = 7.6 Hz, H-2). ^{13}C-NMR (including DEPT and HMQC) spectrum of **3** exhibited 25 carbon signals consisting of a carbonyl carbon at δ 174.3, 22 methylene carbons (δ 22.7–34.2), one methene carbon (δ 70.2), one methyl carbon (δ 14.1) and three oxygenated carbons [δ 70.2 (C-2′), δ 65.2 (C-1′) and δ 63.3 (C-3′)]. The ^{1}H- and ^{13}C-NMR spectral data of **3** were similar to those of sn-1,2-D-α-linoleate-3-palmitate (Enwerem et al., 2001; Herz et al., 1972). The difference between the ^{1}H-NMR of sn-glycerol-1,2-D-α-linoleate-3-palmitate and that of **3** lies in the fatty acid esterifying the primary hydroxyl of the glycerol. The ^{1}H-NMR of **3** revealed methylene proton signals centred at δ 1.26 ppm, integrated to 44 protons, suggesting behenic acid [$C_{22}H_{45}COOH$] as the straight chain fatty acid esterifying the glycerol. The DEPT spectrum of **3** further confirmed 22 methylene carbons (Table 1). Besides, the ^{1}H COSY correlations among the alkoxy protons in the region δ 4.21–3.62, in conjunction with the HSQC correlations of these protons and with the carbon bearing them, revealed the presence of a glycerol moiety [δ_H 4.21 (1H, dd J = 7.02, 4.2 Hz H-1′), δ_H 4.18 (1H dd, J = 7.02, 5.94 Hz, H-1′), δ_H 3.94 (1H, m, H-2′), δ_H 3.72 (1H dd, J = 7.86, 5.62 Hz, H-3′) and δ_H 3.62 (1H dd, J = 7.86, 5.6 Hz, H-3′)]; and corresponding carbon signals at δ_C 70.2 (C-2′), 65.2 (C-1′) and 63.3 (C-3′). Long-range correlations of carbonyl carbon at δ 174.3 with the oxymethylene protons at δ 4.21 and 4.18 confirmed attachment of the carbonyl at C-1 of the glycerol moiety. The ^{1}H COSY and HMBC data revealed that a linear chain of 20 methylenes was directly attached to the carbonyl carbon. The position of the carbonyl carbon (C-1) of the ester was further confirmed from the analysis of mass fragmentation in the HR-ESI-MS data, observed at *m/z* 357.1030 ($C_{22}H_{43}O_2$). Furthermore, a methyl group was placed at the terminus of the molecule by a combined ^{1}H–^{1}H COSY and HMBC analyses. The configuration at C-2 was established from the positive optical rotation, which is a general feature of the long-chain 1-*O*-acyl-sn-glycerols (Quingchun et al., 2003).

Compound **1** was identified as 3β-hydroxyl-lup-20(29)-ene-28-oic acid (betulinic acid) on the basis of ^{1}H and ^{13}C-NMR spectral data, as well as comparison of these data with published values. Compound **1** is a pentacyclic triterpenoids, identified in various plant tissues (Robinson and Martel, 1970; Herz et al., 1972; Mulholland et al., 1994; Pisha et al., 1995; Angelica et al., 2005). Compound **2** was characterised and named sitosteryl-3-*O*-β-D-glucopyranoside. Identification of **2** was based on ^{1}H- and ^{13}C-NMR spectral, compared with physical data in the literature (Miemanang et al., 2006).

The known monoacyl glyceride 1-*O*-(13-methyltetradecanoyl)-sn-glycerol (**4**, 300 mg) and 1-*O*-pentadecanoyl-sn-glycerol (**5**, 370 mg) were identified by comparing the physical

properties, ^{1}H-NMR and MS data with the literature values (Quingchun et al., 2003). The configuration at C-2 in **4** and **5** was established from the positive optical rotation, which is a general feature of the long-chain 1-*O*-acyl-sn-glycerols (Quingchun et al., 2003).

Anthelmintic activities of the extracts and isolated compounds were evaluated against *F. gigantica* and *Taenia solium* parasitic worms. The MeOH and EtOAc extracts of *B. confusa* displayed significant anthelmintic activity (Table 2). The MeOH extract is more active than the EtOAc extract. Isolated compounds **1–5** exhibited remarkable anthelmintic activity when compared with the anthelmintic drug, piperazine citrate (Table 2). Betulinic acid (**1**) exhibited the highest activity with mean paralysis and death time of 130 and 180 min, respectively, on *Taenia solium*. The least anthelmintic activity was observed in compound **3** with mean paralysis and death of 20.01 and 30.99 min on *F. gigantica*. The anthelmintic potencies of the tested compounds compared favourably with standard drug used in this assay (Table 3). The function of worm expeller like piperazine citrate is to cause paralysis of the worms such that they are expelled in the faeces of men and animals. The extracts and the isolated compounds from *B. confusa* demonstrated this property and at the same time, caused the death of parasitic worms assayed. Compounds **1–5** were evaluated for *in vitro* cytotoxic activity against a small panel of three human tumour cell lines (Table 4) using MTT assay method with slight modifications (Voorhoeve, 1965). The result of the cytotoxic assay revealed that betulinic acid (**1**), sitosteryl-β-D-glucoside (**2**) displays moderate cytotoxic activity against Helal cancer cell lines [IC_{50} values of 18.1 and 14.2 μM, respectively]. Isolated monoglyceride (**3–5**) displayed weak cytotoxicity. Compounds **1–5** exhibited weak inhibitory actions against A-549 cell lines with IC_{50} greater than 150 μM. In the case of HL-60, isolated compounds displayed moderate cytotoxicity, IC_{50} ranging from 14.2 to 22.5 μM.

4. Conclusion

In conclusion, three monoacyl glycerols, a triterpenoid and a sitosteryl glucoside were isolated from EtOAc extracts of *B. confusa*, including one new compound. A chemical structure of the new compound was elucidated on the basis of spectroscopic studies. The extracts and isolated compounds displayed dose dependent anthelmintic properties. These results lend support to the traditional usage of *B. confusa* for the treatment of worm infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jscs.2011.11.016.

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