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Research article

In vitro activities of crude extracts and triterpenoid constituents of *Dichapetalum crassifolium* Chodat against clinical isolates of *Schistosoma haematobium*

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ABSTRACT

Dichapetalum crassifolium Chodat (Dichapetalaceae) is widely distributed in Africa, Tropical Asia and Latin America. As part of our quest for potential bioactive lead compounds for various neglected tropical diseases, we report the anti-schistosomal potential of the crude extracts and chemical constituents of the stems and roots of *Dichapetalum crassifolium.* Column chromatography of extracts of the stems and roots led to the isolation and identification of three oleanane-type triterpenoids, friedelan-3β-ol (1), friedelan-3-one (2), and maslinic acid (3); the ursane-type tritepenoid, pomolic acid (4) and the dammarane-type tetracyclic triterpenoids, dichapetalin A (5) and dichapetalin M (6). Dichapetalin A was isolated from only the roots. Isolated compounds were identified by comparison of their physico-chemical and spectral data with published data. The highest *in vitro* anti-schistosomal activity (IC_{50}) of the crude extracts against clinical isolates of *Schistosoma haematobium* (Bilharz 1852) was 248.6 μg/ml for the ethyl acetate extract of the root while dichapetalin A gave the highest activity at 151.1 μg/ml among the compounds compared with the 15.5 μg/ml for the standard drug, praziquantel. The rest of the compounds showed activities in the order 177.9, 191.0, and 378.1 μg/ml respectively for mixture of β-sitosterol/stigmasterol, dichapetalin M and friedelan-3-one. The least active extract was the methanol extract of the stem (893.7 μg/ml). The constituents of *D. crassifolium* showed activity against the *S. haematobium* that are below praziquantel. It is envisaged that the presence of multiple layers and the minute sizes of pores in the egg shells, may preclude penetration of eggs by the compounds.

1. Introduction

Naturally-occurring pentacyclic triterpenoids of the lupane, oleanane and ursane classes are known to possess a variety of biological activities. Quite a number of these triterpenoids have been isolated and identified from some plant species of the Dichapetalaceae family. One of the hitherto uninvestigated species is *Dichapetalum crassifolium* Chodat widely distributed in Africa, Tropical Asia and Latin America (Breteler, 1978). It is typically found in the rain or gallery forests, primitive woods, shady places, and among rocks (Breteler, 1978; Hiern et al., 1901). Even though there is no documented ethnobotanical use and phytochemical investigation for *D. crassifolium,* other species of the genus have indicated the presence of a wide range of secondary metabolites with diverse biological activities. These include the fluorinated carboxylic acids reputed to be responsible for the toxicity of some members of the genus (Meyer and O'Hagan, 1992) as well as various types of triterpenoids including the dichapetalins reputed to have cytotoxic and antiproliferative activities (Fang et al., 2016; Long et al., 2013; Osei-Safo et al., 2012). Other non-terpenoidal compounds recently reported from the genus are the bisbenzyl derivatives heudelotol A and B from *D. heudelotii* (Osei-Safo et al., 2017). Additional compound types obtained from the genus are the alkaloid trigonelline, the amino acids N-methylserine and N-methylalanine (Breteler, 1978; Eloff, 1980), sugars, various glycosides, esters of (E)-ferulic acid (Addae-Mensah et al., 2007; Adu-Kumi, 1997) and pyracrenic acid (Long et al., 2013).

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Apart from the cytotoxic and antiproliferative activities exhibited by the dichapetalins (Achenbach et al., 1995; Addae-mensah et al., 1996; Jing et al., 2014; Osei-Safo et al., 2017), this unique class of triterpenoids has also shown anthelmintic (Chama et al., 2015; Jing et al., 2014), antifungal, feeding deterrent, inhibition of intracellular release of nitric oxide (NO) and acetylcholinesterase (AChE) activities (Jing et al., 2014). As part of our quest for potentially active constituents against parasitic and other causative agents of various neglected tropical diseases, we report the anti-schistosomal activity of the crude extracts and constituents of the stems and roots of the hitherto uninvestigated *D. crassifolium* against clinical isolates of *Schistosoma haematobium* Bilharz 1852, (Tan and Ahana, 2007).

2. Materials and methods

2.1. Materials

The roots and stems of *D. crassifolium* were obtained from the Bobiri Forest Reserve in the Bosomtwe district of the Ashanti Region in July 2013. Identification was done by John Ntim-Gyakare formerly of the Forestry Commission, Kumasi. Voucher specimen (DCR001) has been deposited in the Ghana Herbarium, Department of Plant and Environmental Biology, University of Ghana.

TLC was performed on aluminium foil slides pre-coated with silica gel (thickness 0.2 mm, type Kieselgel 60 F_{254}, Merck, Rogers, AR); detection: I_2 vapour, vanilin stain and anisaldehyde spray reagent. Column chromatography was carried out on silica gel 60 (Fluka Analytical, Bellefonte, PA). Melting points (uncorrected) determined on a Stuart Scientific Melting Point Apparatus (Sigma Aldrich, St. Louis, MO). IR spectra were obtained on an FT IR spectrometer at the Food and Drugs Authority in Ghana. Visualisation of spots under UV light was done with UVGL-58 Handheld UV lamp at 254–365 nm. Organic solvents were concentrated using Buchi Rotary Vacuum Evaporator.

NMR were run on a 500 or 600 MHz Brüker Avance instrument at 90, 125 or 150 MHz for ^{13}C NMR and 360, 500 or 600 MHz for ^{1}H NMR. Depending on the solubility of a particular compound, solvents used were $CDCl_3/CD_3OD$, DMSO-d6, acetone-d6 or CD_3OD with TMS as the internal standard. Schistosomal activity testing was carried out at the parasitology laboratory of the Noguchi Memorial Institute for Medical Research, University of Ghana. Ethical clearance for the anti-schistosomal work was obtained from the Noguchi Memorial Institute for Medical Research (NMIMR-IRB CPN 059/13–14). Schistosome egg recovery and concentration from infested urine samples was by the modified Kotze et al. method (Kotze et al., 2005). Urine reagent strips were obtained from URIT Medical Electronic Co, Ltd, China. Different pore sizes of sieves for filtration of suspensions were obtained from Nonaka Rikaki Co. Ltd, Japan.

2.2. Methods

2.2.1. Sample collection of clinical isolates

S. haematobium eggs were obtained from urine samples collected from 120 school children in Tomefa in the Ga South District of Accra and stored in air-tight plastic containers. More than 80% of participants were between ages 8 and 14 years. Samples were first tested with urine reagent strips, URIT 10V to identify cases of haematuria and then kept in a Styrofoam box and transported to the parasitology laboratory at the Noguchi Memorial Institute for Medical Research.

2.2.2. Recovery, purification and identification of S. haematobium eggs from urine samples

Portions (10 ml) of collected urine samples were centrifuged and observed using a low power microscope to determine the presence of *S. haematobium*, whose eggs were identified by the presence of terminal spines (Figure 7). Urine samples that were positive for schistosome eggs were pooled into 50 ml falcon tubes and centrifuged at 500x*g* for 5 min. The supernatant was discarded and the deposits suspended in 50 ml of normal saline (0.9%, NaCl) and centrifugation repeated. The sediment was suspended in 40 ml of 0.015% Brij-35 and shaken vigorously. It was centrifuged again at 500x*g* for 5 min. The supernatant was discarded and the sediment re-suspended in normal saline into a 200 ml beaker. More saline was added to make up to the 150 ml mark. Due to the different particle sizes of faecal samples which do not usually produce clean egg cells of the parasite, sieves of different pore sizes were used.

For differential separation to obtain cleaner schistosome eggs, the suspension was filtered through a stack of three sieves of pore size 180 μm, 150 μm and 80 μm respectively.

The stack of sieves was thoroughly flushed with a jet of normal saline to wash the eggs. To prevent air lock, the 150 μm and 80 μm sieves were occasionally separated. At the end of washing, the 80 μm sieves were removed and inclined at approximately 45° to the horizontal and a jet of saline applied to it to wash off the eggs into a beaker. The suspension was centrifuged at 200x*g* for 10 min and left for 30 min after which the supernatant was aspirated down to 5 ml. Three 30 μl suspensions of the concentrated schistosome eggs were pipetted and observed under light microscope, the number of eggs per 30 μl was noted and hence the average number of eggs per 30 μl determined. From this result, the approximate number of *S. haematobium* eggs in the 5 ml concentrated egg solution was calculated.

2.3. Extraction, isolation and structure elucidation of compounds

Soxhlet extraction of 5 kg each of pulverized roots and stems in batches of 500 g of *D. crassifolium* was carried out exhaustively using 5 L of petroleum ether for 24 h to give 54 g root and 34 g of stem extracts after concentration, Figure 1. Each plant residue was further extracted with EtOAc and MeOH respectively after drying. Concentration of the root extracts gave 77 g (EtOAc extract) and 108 g (MeOH extract) crude material. The stem yielded 65 g (EtOAc extract) and 23 g (MeOH extract) of crude material, Figure 1.

Column chromatography of the petroleum ether extract of the stem (20 g) on 230 g silica gel, eluting with petroleum ether/EtOAc (10:1 to 0:10, v/v) gave three main fractions, S1–S3, Figure 2. Fraction S2 (12.6 g) was re-chromatographed on silica gel (80 g) with petroleum ether/$CHCl_3$ (1:3, v/v) to give three fractions (S4–S6). Fraction S4 gave compounds 1 (180 mg), while fraction S6 yielded compound 2 (80 mg), both as white crystals, as well as a mixture of the two compounds. Similar separations of the petroleum ether extract of the roots also gave compounds 1 (215 mg), and 2 (82 mg) (Figure 2). The EtOAc extract (40 g) of the stem was also column chromatographed on silica gel (450 g) with petroleum ether/EtOAc solvent gradient (10:1 to 0:10, v/v) to give five main fractions S7–S11. Fraction S7 yielded compounds 1 and 2 identified respectively as friedelan-3β-ol and friedelan-3-one (Figures 3 and 4) while S8 gave a mixture of β-sitosterol/stigmasterol (7 and 8, 25 mg), Figures 4 and 5. Fraction S9 precipitated solids recrystallized from a mixture of $Et_2O/CHCl_3$ (10:3, v/v) to afford a white powdery compound 4 identified as pomolic acid (60 mg, Figure 3). Fraction S11 was re-chromatographed in similar manner to afford compounds 6 (dichapetalin M, 20 mg) as a white amorphous solid, recrystallized from a mixture of $Et_2O/CHCl_3$ (2:5, v/v), and compound 3, maslinic acid (Figure 3) as a creamy powder (32 mg) recrystallized from a mixture of $Et_2O/CHCl_3$ (2:5, v/v), Figure 4. Similar separation procedures by column chromatography were also carried out on the crude EtOAc extract of the roots to yield compounds 3, 4, 5 and 6. Column chromatography of the EtOAc extract (38 g) on 450 g silica gel, eluting with petroleum ether/EtOAc solvent gradient (10:0 to 0:10, v/v) gave nine main fractions R1-R9. Fractions R1-R2 gave compound 3, while compound 4 was obtained from fraction R3 and compound 6 from fraction R4. Compound 5, 40 mg, was isolated from fraction R7 and recrystallized from Et_2O/EtOAc (10:1, v/v) as yellowish flakes (Figure 4). All compounds were characterised by comparison of their physico-chemical properties (melting point, TLC

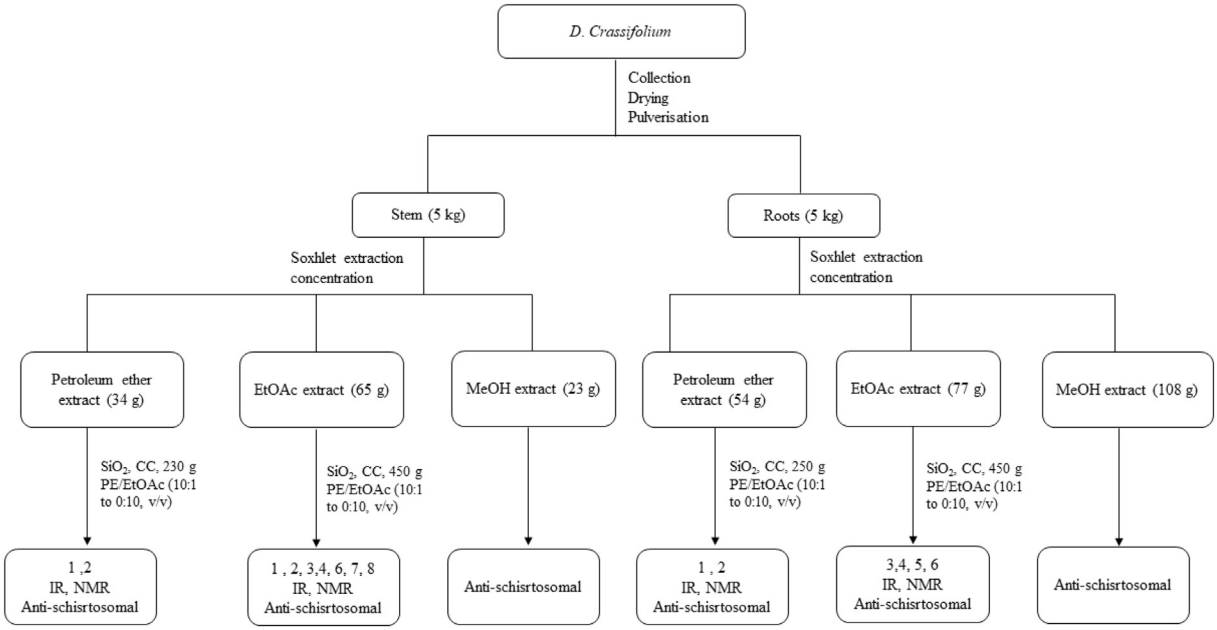

Figure 1. Flow diagram of the work done on petroleum ether, ethyl acetate and methanol extracts of the stem and root of *D. crassifolium*. PE: Petroleum ether.

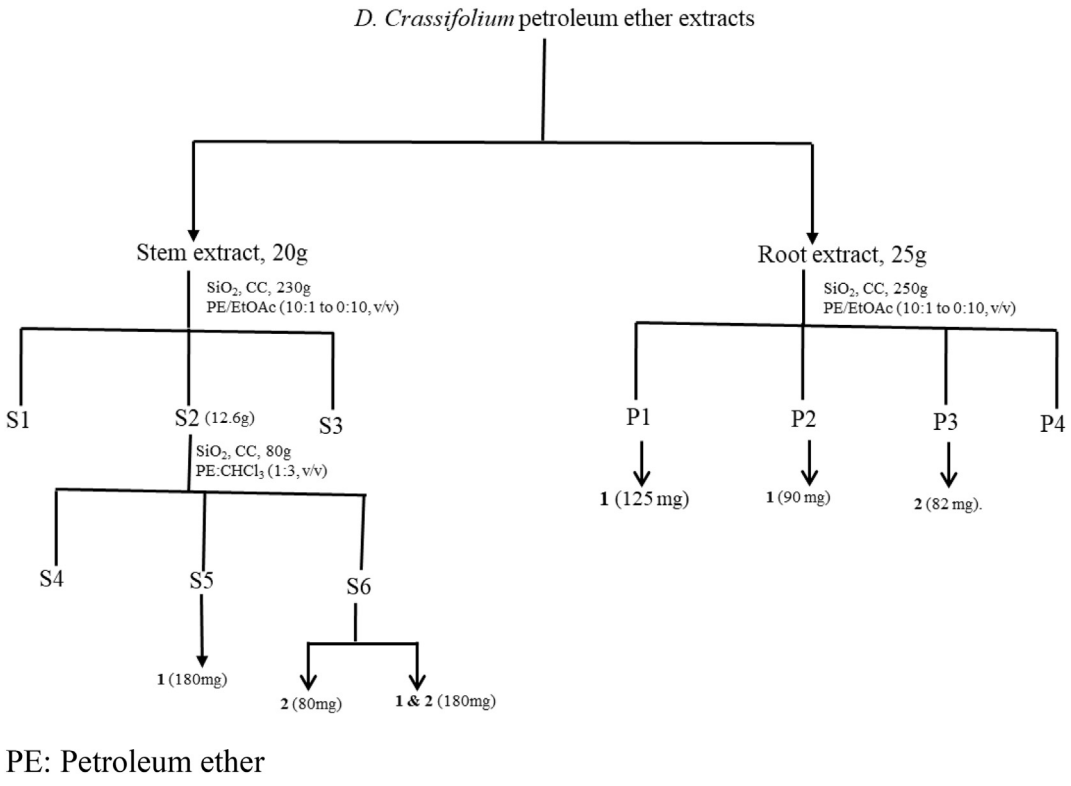

Figure 2. Chromatographic separation of the petroleum ether extracts of the stem and root of *D. crassifolium*.

profile in various solvent systems), as well as their spectroscopic data (IR, ^{1}H and ^{13}C NMR) with literature data as indicated below.

2.3.1. Physico-chemical properties and spectral data of isolated compounds

(3S,4R,4aS,6aS,6aS,6bR,8aR,12aR,14aS,14bS)-4,4a,6a,6b,8a,11,11,14a-octamethyl-1,2,3,4,5,6,6a,7,8,9,10,12,12a,13,14,14b-hexadecahydropicen-3-ol (*Friedelan-3β-ol*, **1):** 53 mg as white crystals; mp: 272–274 °C (Lit. 274–276 °C, (Utami et al., 2013); Anisaldehyde: purple; IR ν_{max} (KBr) cm^{-1}; 3477 (O–H), 2932, 2871 (C–H), 1448, 1385 [$C(CH_3)_2$]; Co-TLC with authentic sample in 100% $CHCl_3$, R_f 0.81; petroleum ether/EtOAc (14:0.5), R_f 0.61; and petroleum ether/Me_2CO (12:0.5), R_f 0.5.

4,4*a*,6*a*,6*b*,8*a*,11,11,14*a*-octamethyl-2,4,5,6,6*a*,7,8,9,10,12,12*a*,13,14,14*b*-tetradecahydro-1*H*-picen-3-one (*Friedelan-3-one,* **2**): 215 mg as white crystals; mp: 249–251 °C (Lit. 249–251 °C, (Utami et al., 2013); Anisaldehyde: yellow; IR ν_{max} (KBr) cm^{-1}; 2927, 2870 (C–H), 1716 (C=O), 1462, 1389 [$C(CH_3)_2$]; Co-TLC

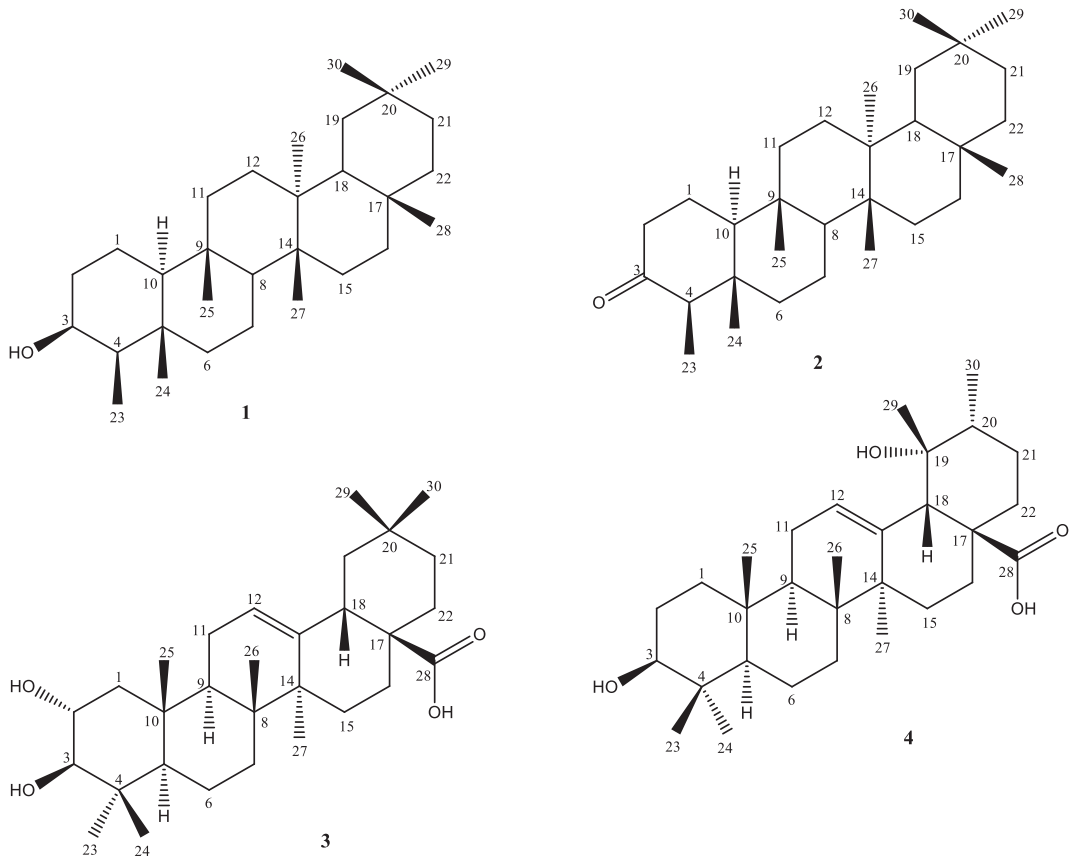

Figure 3. Structures of friedelan-3β-ol **1,** friedelan-3-one **2,** maslinic acid **3** and pomolic acid **4**.

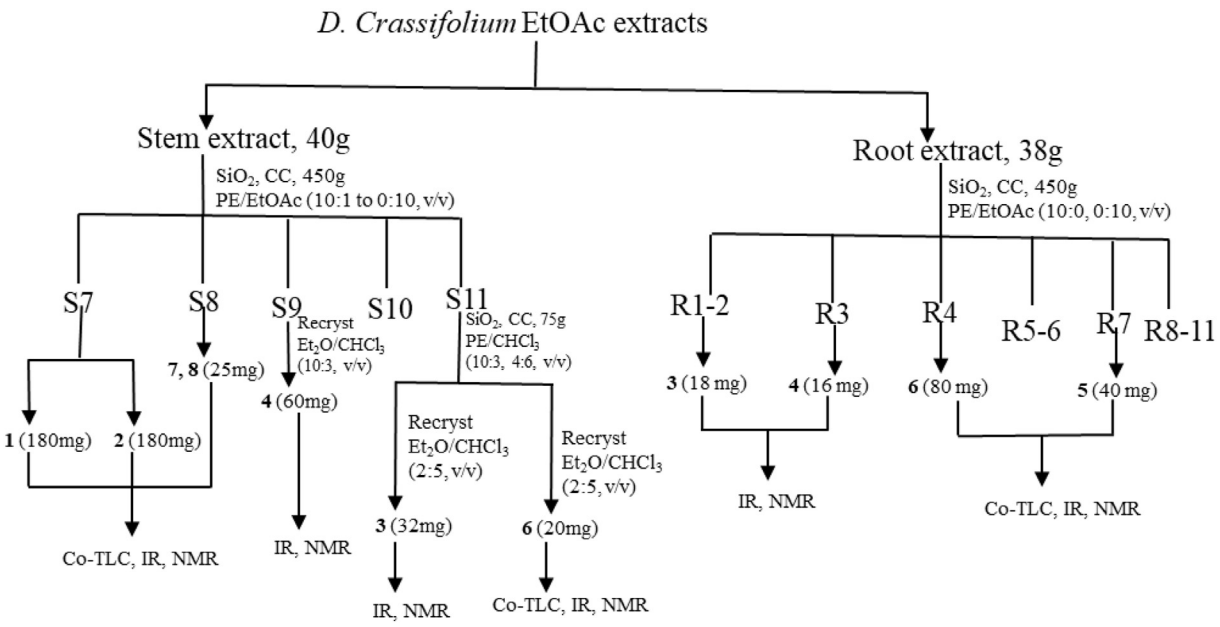

Figure 4. Chromatographic separation of the ethyl acetate extracts of the stem and root of *D. crassifolium*. PE: Petroleum ether.

with authentic sample in 100% $CHCl_3$, R_f 0.94; petroleum ether/EtOAc (14:0.5), R_f 0.87; and petroleum ether/Me_2CO (12:0.5), R_f 0.82.

(2α,3β)-2,3-Dihydroxyolean-12-en-28-oic acid (*Maslinic acid,* **3**), 50 mg as creamy powder: mp: 249–251 °C (Lit. 248–250 °C, (Hossain and Ismail, 2013); Anisaldehyde: purple; IR ν_{max} (KBr) cm^{-1}; 3443 (O–H), 2941 (C–H), 1692 (C=O), 1461, 1385 (C–H), 1270 (C–O–H) and 1051.37 (C–O). ^1H and ^{13}C NMR: see Table 1 (Woo et al., 2014) and Supplementary material Figures S1 and S2. TLC: petroleum ether/Et_2O (3:7), R_f 0.68; petroleum ether/EtOAc (10:3), R_f 0.53; petroleum ether/Me_2CO (10:3), R_f 0.40; petroleum ether/EtOAc (10:6), R_f 0.88.

(1*R*,2*R*,4*aS*,6*aR*,6*aS*,6bR,8*aR*,10*S*,12*aR*,14*bS*)-1,10-dihydroxy-1,2,6*a*,6*b*,9,9,12*a*-heptamethyl-2,3,4,5,6,6*a*,7,8,8*a*,10,11,12,13,14*b*-

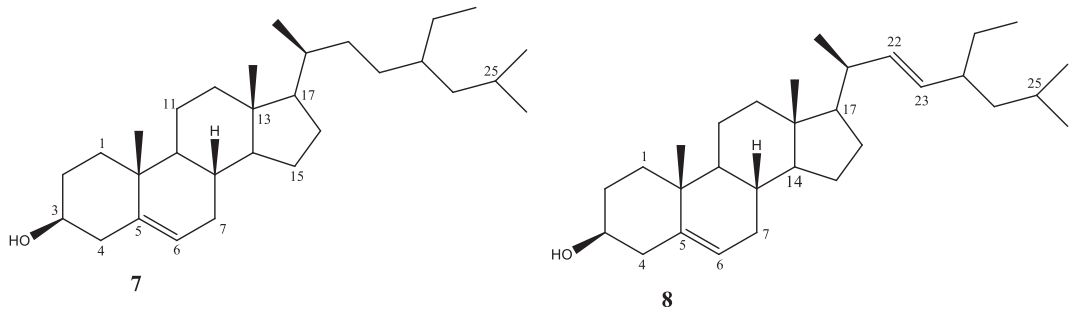

Figure 5. Structures of β-sitosterol (7) and stigmasterol (8).

Table 1. ^{1}H and ^{13}C NMR data of maslinic acid and pomolic acid isolated from *D. crassifolium*.

Position	Maslinic acid **(3)** ($CD_3OD/CDCl_3$)		Pomolic **(4)** acid (Acetone-D6)	
	delta;$_C$/ppm	δ_H (J, Hz) (Woo et al., 2014)	δ_C/ppm	δ_H (J, Hz) (Lee et al., 2005; Zhu et al., 2012)
1	47.7	-	39.5	-
2	68.8	3.66(1H, ddd, $J_1 = 10.3$, $J_2 = 8.5$, $J_3 = 6.5$ Hz)	27.5	2.63(2H, ddd, $J_1 = 8.8$, $J_2 = 9.6$, $J_3 = 6.6$ Hz)
3	83.7	3.45(1H, d, 10.3 Hz)	78.3	3.15(1H, dd, $J_1 = 4.5$, $J_2 = 10$ Hz)
4	39.3	-	39.5	-
5	55.4	-	53.0	-
6	18.5	-	18.0	-
7	32.7	-	32.4	-
8	39.4	-	38.3	-
9	49.0	1.90(1H, dd, $J_1 = 4.2$, $J_2 = 7.8$ Hz)	48.2	2.55(1H, s)
10	38.3	-	37.2	-
11	23.6	1.61(2H, dd, $J_1 = 11.3$, $J_2 = 11.3$ Hz)	23.8	-
12	122.2	5.27(1H, t, J = 3.5 Hz)	128.5	5.28(1H, t)
13	144.1	-	137.8	-
14	42.0	-	40.9	-
15	27.8	-	29.2	-
16	23.1	1.95(2H, ddd, $J_1 = 11.6$, $J_2 = 9.6$, $J_3 = 9.6$ Hz)	26.3	1.95(2H, d, J = 5 Hz)
17	46.5	-	47.8	-
18	41.3	2.83(1H, dd, $J_1 = 7.8$, $J_2 = 8.3$ Hz)	54.8	2.80(1H, s)
19	46.3	-	72.5	-
20	30.8	-	40.8	-
21	34.0	-	26.4	-
22	33.2	-	38.1	1.94(2H, d, J = 4.6 Hz)
23	28.7	1.02(3H, s)	27.8	2.55(3H, s)
24	16.6	0.78(3H, s)	16.0	2.25(3H, s)
25	16.8	0.98(3H, s)	15.1	1.35(3H, s)
26	17.0	0.81(3H, s)	18.1	1.21(3H, s)
27	25.7	1.06(3H, s)	25.1	0.99(3H, s)
28	180.8	-	180.5	-
29	32.7	0.90 (3H, s)	25.6	0.95 (3H, d, J = 6.5 Hz)
30	23.6	0.93 (3H, s)	15.5	0.79 (3H, d, J = 6.5 Hz)

tetradecahydropicene-4*a*-carboxylic acid (*Pomolic acid*, **4**): 71 mg as white powder: mp: 268–270 °C (Lit. 276–278 °C); Vanillin stain: purple; anisaldehyde: violet; IR ν_{max} (KBr) cm^{-1}; 3567, 3419 (O–H), 2937, 2874 (C–H), 1686, 1618 (C=O); ^{1}H and ^{13}C NMR: See Table 1 (Chama et al., 2015; Lee et al., 2005; Zhu et al., 2012) and Supplementary material Figures S3 and S4; TLC: petroleum ether/Et_2O (3:7), R_f 0.81; petroleum ether/EtOAc (10:3), R_f 0.45; petroleum ether/Me_2CO (10:3), R_f 0.29; petroleum ether/EtOAc (10:6), R_f 0.77; petroleum ether/EtOAc (1:1), R_f 0.71.

(3*S*,5*R*)-5-[(*E*)-3-hydroxy-2-methylprop-1-enyl]-3-[(1*S*,2*R*,3*R*,5*R*,6*R*,9*S*,14*S*,15*R*,18*S*,19*S*)-3-hydroxy-2,6,14-trimethyl-9-phenyl-8-oxahexacyclo[16.3.1.$0^{1,18}$.$0^{2,15}$.$0^{5,14}$.$0^{6,11}$]docosa-11,16-dien-19-yl]oxolan-2-one (*Dichapetalin A*, **5**): 40 mg as yellow flakes; mp: 210–213 °C (from Et_2O/EtOAc (10:1, v/v)) (Lit.: 212–213 °C, Osei-Safo et al., 2008); Vanilin stain: blue; Anisaldehyde: purple; IR ν_{max} (KBr) cm^{-1}: 3573, 3552, 3369 (O–H); 2957, 2932, 2873 (C–H); 1746 (C=O), 1655 (C=C), 1096, 1039 (C–O–O), 767 (aromatic C–H); ^{1}H and ^{13}C NMR: see Table 2 (Chama et al., 2015; Osei-Safo et al., 2008) and

Table 2. ^{1}H and ^{13}C NMR data of dichapetalins A and M isolated from *D. crassifolium*.

Position	Dichapetalin A (5)		Dichapetalin M (6)	
	δ_C (ppm)	δ_H (*Mult*)	δ_C (ppm)	δ_H (*Muit*)
1(CH_2)	41.3	2.15, s	39.9	2.90,1.65
2(CH)	119.3	5.45, m	116.6	5.40
3(C)	141.2	-	141.3	-
4(C)	39.5	-	39.8	-
5(CH)	43.5	2.00[a]	59.1	1.60
6(CH_2)	24.2	1.87,m	71.3	4.60,dd,3.80
7(CH)	73.2	3.92 (t, J = 1.5)	213.7	-
8(C)	37.4	-	47.0	-
9(CH)	45.1	1.75 (d, J = 2.5)	52.1	2.00
10(C)	37.2	-	36.9	-
11(CH)	124.1	5.43 (dd, J = 10, 2.5Hz)	120.1	5.40,dd
12(CH)	131.1	6.25 (dd, J = 10, 3Hz)	131.6	6.35,dd
13(C)	30.6	-	31.6	-
14(C)	37.5	-	36.5	-
15(CH_2)	26.2	2.04[a]	26.7	2.00,2.20
16(CH_2)	23.9	1.13, s	23.6	1.30,1.80,m
17(CH)	41.8	2.62, m	40.5	2.55
18(CH_3)	18.4	0.94,s	17.1	1.10
19(CH_3)	18.6	-	18.2	1.30,s
20(CH)	42.6	3.30,m	47.3	3.00,dd,2.45
21(C)	181.3	-	174.0	-
22(CH_2)	31.4	2.37,m	72.1	4.20,dd
23(CH)	77.1	5.26, m	111.3	-
24(CH_2)	123.1	5.50, s	45.7	2.90,2.50,d
25(C)	143.2	-	85.1	-
26(CH_2)	67.3	3.98,s	78.5	4.35,d,4.10,d
27(CH_3)	14.1	1.75, s	22.0	1.70,s
28(CH_3)	24.7	1.32, s	26.5	1.55,s
30(CH_2)	16.9	0.85 (d, J = 5Hz)	14.4	1.30,d,1.05
2′ (CH_2)	73.6	3.56 (d, J = 10.5Hz)	71.3	4.35,3.95,d
5′ (CH_2)	42.6	2.20 (dd, J = 13.5, 4Hz)	40.1	2.70, t_{br},2.25
6′ (CH)	83.6	4.27 (dd, J = 11.5, 2.5Hz)	81.3	4.35,dd
1″(C)	143.90	-	142.5	-
2″/6″ (CH)	126.97	7.37, m	128.4	7.45,d
3″/5″ (CH)	129.5	7.33, m	125.7	7.40,t
4″ (CH)	128.5	7.26, m	127.5	7.30,t
AcCO(C)			170.4	-
$AcCH_3(CH_3)$			21.8	2.00,s

[a] Overlapping signals.

Supplementary material Figures S5 and S6; Co-TLC with authentic sample in petroleum ether/Me_2CO (10:3), R_f 0.40; petroleum ether/EtOAc (10:4), R_f 0.65; petroleum ether/EtOAc (10:6), R_f 0.20.

*(3R,8R,9R)-9-hydroxy-8-((3S,6aR,*6bR*,8aS,9S,11aS,*11bR*,13-R,13aR,*13bR*)-13-hydroxy-6a,11b,13b-trimethyl-12-oxo-3-phenyl-1,3,4,6,6a,6b,9,10,11,11b,12,13,13a,13b-tetradecahydro-8a,11a-methanocyclopenta[5,6]naphtho[1,2-h]isochromen-*9-yl*)-3-methyl-7-oxo-1,6-dioxaspiro[4.4]nonan-*3-yl *acetate* (*Dichapetalin M*, **6**), 201 mg as white amorphous solids; mp: 282–284 °C (Lit.: 280–282 °C, (Chama et al., 2015); Vanilin stain/anisaldehyde: violet; IR ν_{max} (KBr) cm^{-1}: 3432, 1244 (O–H); 2976, 2883, 1367 (C–H); 1770, 1751, 1700 (C=O), 1618 (C=C), 1191 (C–O–O), 671 (aromatic C–H) cm^{-1}; ^{1}H and ^{13}C NMR: see Table 2 (Chama et al., 2015; Osei-Safo et al., 2008) and Supplementary material Figures S7 and S8. Co-TLC with authentic sample in $Et_2O/CHCl_3$ (2:5), R_f 0.48; petroleum ether/EtOAc (10:3), R_f 0.38; petroleum ether/EtOAc (10:6), R_f 0.67 (Osei-Safo et al., 2008).

17-(5-Ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1H-cyclopenta[a]phenanthren-3-ol/(3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[a]phenanthren-3-ol (*β-Sitosterol/stigmasterol*, **7**, **8**); 25 mg as white crystals; mp: 138–140 °C (Lit. 142–145 °C, (Chama, 2007); Anisaldehyde: orange; IR ν_{max} (KBr): 3434 (O–H); 2936, 2882, 1463, 1382, 1062 (C–H); 1639, 1618 (C=C) cm^{-1} (Chama, 2007); Co-TLC with authentic sample in 100% $CHCl_3$, R_f 0.50; petroleum ether/EtOAc (10:4), R_f 0.82; petroleum ether/Me_2CO (12:0.5), R_f 0.21.

2.4. *In vitro screening of test samples against schistosome eggs using the 96-well plate-egg hatch assay*

For each of the test compounds; namely, friedelan-3-one (2), dichapetalins A (5) and M (6) and mixture of β-sitosterol/stigmasterol (6, 7), 2

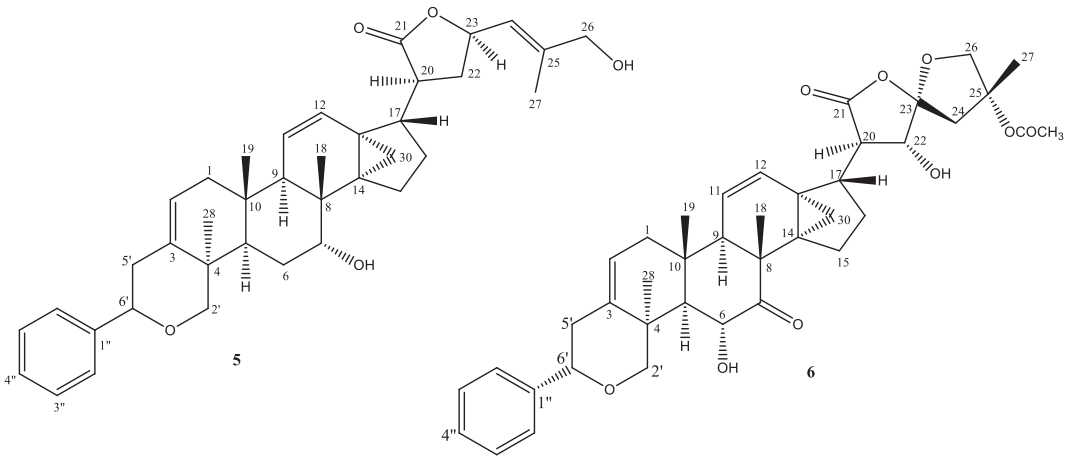

Figure 6. Structures of dichapetalins A (**5**) and M (**6**).

mg was dissolved in 1 ml of 5% DMSO to give 2 mg/ml stock solution. For each of petroleum ether, EtOAc, and MeOH crude extracts of the roots and stems, 5 mg was dissolved in 1 ml of 5% DMSO to obtain a stock solution of 5 mg/ml.

For each test sample of the stock solution, 100 µl was pipetted in duplicate into the first wells of 96-well plate. Serial dilutions of the 2 mg/ml solutions produced 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 mg/ml. Serial dilutions with concentrations 2.5, 1.25, 0.625, 0.3125, 0.15625 and 0.078125 mg/ml of the stock (5 mg/ml) solution of the crude extracts were also prepared. From the suspension of purified eggs, 50 µl was added to each well and the presence of eggs was ascertained under an inverted microscope. The final concentration of DMSO was kept below 0.1%. The choice of DMSO as a solvent and selection of suitable concentration that had no effect on the morphology of the eggs followed the method of Treger et al. (2014). Water and praziquantel (2 mg/ml stock solution) were used as negative and positive controls respectively. Praziquantel, because of its partial solubility in cold water, was dissolved in warm water. The assays were incubated for 24 h at ambient temperature and the process of egg hatch was stopped by the addition of 100 ml of formalin to each well. The number (#) of hatched eggs and unhatched eggs (larvae) was counted using inverted light microscopy.

The percent egg hatch inhibition (% EHI) was calculated as:

$$\%Egg\ Hatch\ Inhibition(\%EHI) = \frac{\#of\ unhatched\ eggs}{\#\ of\ unhatched\ eggs + \#\ of\ hatched\ eggs} \times 100$$

The %EHI was plotted against log of the concentration. Extrapolation of the 50% EHI on the curve gave the half maximal inhibitory concentration (IC_{50}) of each test sample using GraphPad Prism v.7.

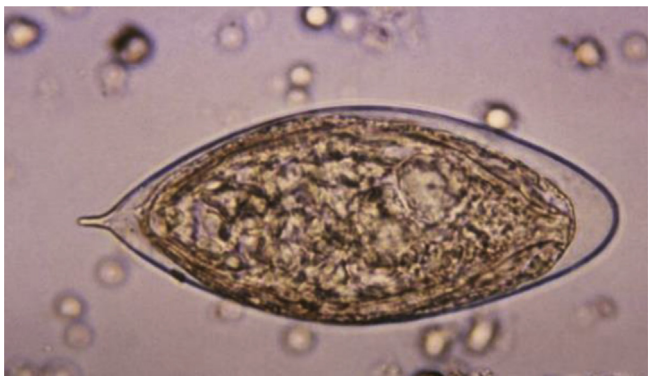

Figure 7. Egg of *Schistosoma haematobium* isolated from school children in Tomefa in the Ga South District of Accra, Ghana under a low power microscope.

3. Results and discussion

Compound **1** was identified as friedelan-3β-ol (Figure 3), mp 272–274 °C (Lit. 274–276 °C, Utami et al., 2013) and stained purple with anisaldehyde spray reagent. Compound **2** was also identified as friedelan-3-one (Figure 3), mp: 249–251 °C, (Lit. 249–251 °C, Utami et al., 2013; Sousa et al., 2012) and stained yellow on TLC with anisaldehyde spray reagent. Other physico-chemical and spectroscopic properties were consistent with those reported in literature (Chama, 2007; Chama et al., 2015; Osei-Safo et al., 2008). Compound **3** was identified similarly as maslinic acid (Figure 3, Table 1), creamy powder which stained purple with anisaldehyde spray reagent, mp 249–251 °C (Lit. 248–250 °C, (Hossain and Ismail, 2013; Lozano-Mena et al., 2014; Tanaka et al., 2003; Woo et al., 2014). This is the first report of maslinic acid from the genus *Dichapetalum* aside its isolation from *Crataegus oxyacantha* and *Eriobotrya japonica*. Compound **4** was characterised as pomolic acid (Figure 3, Table 1). Melting point and spectral data were consistent with literature values (Chama et al., 2015). Compounds **5** and **6** (Figure 6 were each identified as dichapetalin A and M, respectively upon comparison of their physico-chemical and spectral data with literature, Table 2. Melting point of dichapetalin A (210–213 °C) and spectral data (Table 2) were consistent with literature (Lit: 212–213 °C, Achenbach et al., 1995; Addae-mensah et al., 1996; Chama et al., 2015; Osei-Safo et al., 2008). Melting point of dichapetalin M, 282–284 °C and spectral data were also consistent with that of known values (Lit: 280–282 °C, Osei-Safo et al., 2008). This is the first report of the presence of these two dichapetalins in *D. crassifolium* and this could be of chemotaxonomic importance. With the exception of compounds 1 and 2 which were isolated from the petroleum ether extracts, the rest of the compounds including 1 and 2 were isolated from the EtOAc extracts. Phytochemical screening indicated the petroleum ether extracts contained only terpenoids in the stem and terpenoids, saponins and cardiac glycosides in the roots. Also, terpenoids and tannins were present in the EtOAc extracts of both the stem and root extracts in addition to saponins and cardiac glycosides which were present only in the roots. The methanol extracts contained terpenoids, tannins, and cardiac glycosides.

Dichapetalin A was first isolated from *D. madagascariense* (Achenbach et al., 1995) as the major constituent of the roots. Later the compound was isolated from *D. gelonioides* (Fang et al., 2016), *D. ruhlandii, D. mombuttense, D. zenkeri, D. eickii, D. fillicauli* (Chama et al., 2015) and *D. pallidum* (Osei-Safo et al., 2017). Dichapetalin M has so far been isolated from only two other species, *D. Madagascariense* (Osei-Safo et al., 2008) and *D. eickii* (Long et al., 2013). Pomolic acid has also been isolated from *D. fillicauli* (Chama et al., 2015) and *D. pallidum* (Osei-Safo et al., 2017). Earlier isolation of friedelan-3β-ol and friedelan-3-one was reported in *D. barteri*, *D. madagascariense* (Osei-Safo et al., 2008), *D. filicaule* (Chama

Table 3. Half maximal inhibitory concentrations, IC_{50} (μg/ml, ± SEM) of isolated compounds and extracts from the root and stem of *D. crassifolium* compared with praziquantel standard against *S. haematobium* eggs.

Compound/Extract	IC_{50} ± SEM (μg/ml)	
Friedelan-3-one	378.10 ± 0.23	
β-Sitosterol/stigmasterol	177.90 ± 0.10	
Dichapetalin M	191.00 ± 0.12	
Dichapetalin A	151.1 ± 0.21	
Extracts	Root	Stem
Petroleum ether	546.40 ± 0.06	443.70 ± 0.04
EtOAc	248.60 ± 0.10	638.00 ± 0.08
MeOH	566.30 ± 0.07	893.70 ± 0.08
Praziquantel	15.47 ± 0.06	

et al., 2015) and *D. pallidum* (Osei-Safo et al., 2017). β-Sitosterol and stigmasterol have previously been isolated from *D. barteri* and *D. madagascariense*. β-Sitosterol and stigmasterol commonly occur in most plants and differ structurally only at the C22–C23 due to the presence of unsaturation in the latter and as a result both are often isolated together as a mixture.

The most abundant of the tritepenoids were the friedelan-3β-ol and friedelan-3-one. Even though phytochemical screening indicated the presence of terpenoids, tannins and cardiac glycosides in the roots and terpenoids, tannins in the stem of the ethyl acetate extracts, only triterpenoids were isolated suggesting that they constituted the abundant phytochemicals in the plant whiles tannins and cardiac glycosides occurred in least amounts.

3.1. Results of in vitro anti-schistosomal activity against Schistosoma haematobium

The picture of *Schistosoma haematobium* eggs identified under a low power microscope is presented in Figure 7. The half maximal inhibitory concentrations, IC_{50} (μg/ml, ± SEM) of reference standard, compounds and extracts were determined for duplicate experiments (n = 2) as presented in Table 3.

Generally, the isolated compounds showed higher ovicidal activity against *S. haematobium* eggs than the extracts though activities for both samples were low compared to the standard praziquantel (Table 3). Among the compounds isolated from the stem, the mixture of β-sitosterol/stigmasterol showed the highest potency (IC_{50}, 177.90 μg/ml) but this was about 11 times less potent than the standard praziquantel drug (15.47 ± 0.06 μg/ml), (Table 3, Figure 8). The next highest was dichapetalin A (151.10 μg/ml) whiles friedelan-3-one showed the least potency with IC_{50}, 378.10 μg/ml (Table 3, Figures 8 and 9). Dichapetalin M from the root extract gave an IC_{50} of 191.00 μg/ml, (Table 3, Figure 9).

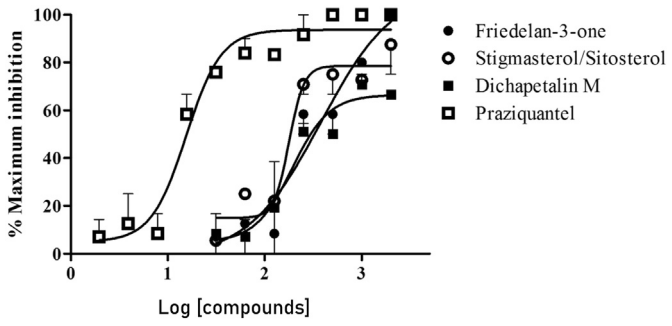

Figure 8. Concentration-dependent effect of friedelan-3-one, stigmasterol/sitosterol and dichapetalin M with standard praziquantel from the stem of *D. crassifolium* against hatching of *S. haematobium* eggs. Each point is ±SEM (n = 2).

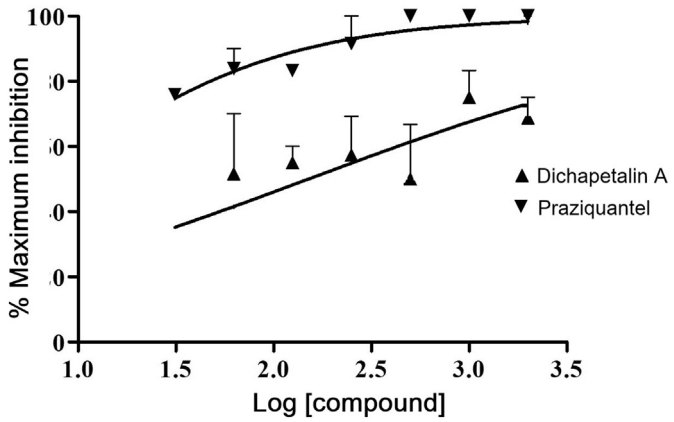

Figure 9. Concentration-dependent effect of dichapetalin A with standard praziquantel from the roots of *D. crassifolium* against hatching of *S. haematobium* eggs. Each point is ±SEM (n = 2).

All the triterpenoids were isolated from the polar EtOAc extracts. This might be the reason why the most active compound, dichapetalin A was isolated from the most active root EtOAc extract. The root crude extracts were generally more active than the stem crude extracts (Table 3) even though the root EtOAc extract contained additional saponins and cardiac glycosides aside the terpenoids and tannins which were also contained in the stem EtOAc extract. This could be because of the roots containing more of the terpenoids as observed in the greater yield of the root extracts (1.5%) compared with the stem extracts (1.3%).

The IC_{50} values for the root extracts were 248.60 (EtOAc), 546.40 (petroleum ether) and 566.30 (MeOH) μg/ml, while the stem crude extracts gave values of 443.70 (petroleum ether), 638.00 (EtOAc) and 893.70 (MeOH) μg/ml (Table 3, Figures 10 and 11).

Against the beet armyworm (*Spodoptera exigua*), dichapetalin A showed antifeedant activity with an EC_{50} of 3.1 μg/cm^2 (Jing et al., 2014). The compound also indicated nematicidal activity of 15.3% rate at 100 μg/ml over 72 h against *Panagrellus redivivus* (Jing et al., 2014) and anti-hookworm activity against *Necator americanus* eggs with IC_{50} of 162.4 μg/ml (Chama et al., 2015). Against parasitic fungi, dichapetalin A was almost as active as nystatin with growth inhibition of 12.8 ± 0.8 and 10.5 ± 0.02 at 50 μg/disk on *Colletotrichum musae* and *Rhizoctonia solani* respectively (Jing et al., 2014).

Except for the anti-shistosomal activity, dichapetalin M has not been tested for its antiparasitic activities. Antiplasmodial activity evaluated for friedelan-3-one (2) against W2 strain of *Plasmodium falciparum* gave an IC_{50} of 7.7 Mm (Ndjakou Lenta et al., 2007). Stigmasterol showed potency on both 3D7 and K1 strains of *Plasmodium falciparum* with EC_{50} of 3

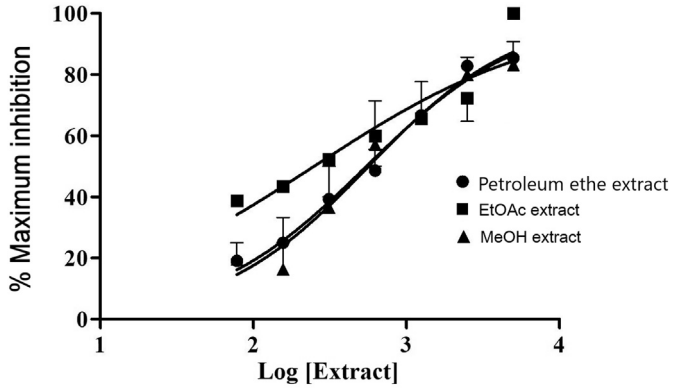

Figure 10. Concentration-dependent effect of petroleum ether, EtOAc and MeOH root extracts of *D. crassifolium* against hatchability of *S. haematobium* eggs. Each point is ±SEM (n = 2).

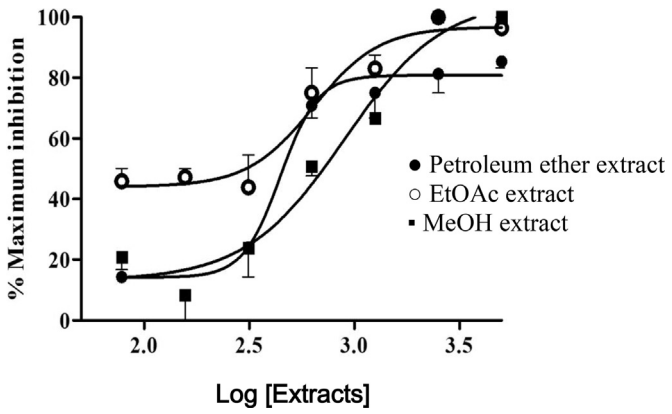

Figure 11. Concentration-dependent effect of petroleum ether, EtOAc and MeOH extracts of the stem of *D. crassifolium* against hatchability of *S. haematobium* eggs. Each point is ±SEM (n = 2).

μM and 5 μM respectively (Zhai et al., 2014). *Beta*-sitosterol indicated IC_{50} of 2.28 ± 0.01 μg/ml against 3D7 strain of *P. falciparum* and against Dd2 strain, it indicated >50 μg/ml (Langat et al., 2019).

A number of terpene compounds including monoterpenes, sesquiterpenes, diterpenes and triterpenes have showed both remarkable *in vitro* and *in vivo* antischistosomal activity. The mechanism of action of their schistosomicidal effects are affected by their lipophilicity that allows easy crossing of the plasma membrane and interaction with intracellular molecules of parasites to cause morphological changes (de Moraes, 2015). Monoterpenes with *in vitro* activity against *S. mansoni* adult worms include, rotundifolone from *Mentha x villosa* at a concentration of 70 μg/ml (Matos-Rocha et al., 2013). (+)-Limonene epoxide in essential oils also showed activity at a concentration of 25 μg/ml together with the detection of morphological alterations on the schistosome surface at concentrations of 25–75 μg/ml (de Moraes et al., 2013). Among the antischistosomal sesqueterpene compounds active against the immature stages of *S. mansoni* are the antimalaria compounds artemisinin from the leaves of *Artemisia annua* L., artemether, artesunate and dihydroartemisinin (Liu et al., 2014; Utzinger et al., 2001). The essential oil nerolidol was effective *in vitro* against adult worms of *S. mansoni* with a reduction in worm motor activity death at 31–62 μM (Silva et al., 2014). Budlein-A from *Viguiera spp* (Asteraceae) showed *in vitro* schistosomicidal activity against *S. mansoni* adult worms at 12.5 μM and its derivatives 4α, 5-dihydrobudlein A and 4α,5-11β,13-tetrahydrobudlein A gave 100% mortality at the respective concentrations of 50 and 200 μM (Sass et al., 2014). The diterpene phytol, from chlorophyll has also indicated promising *in vitro* and *in vivo* activity with infected mice against adult *S. mansoni* (de Moraes et al., 2014). Aside these, triterpenes such as betulin isolated from *Schefflera vinosa* plant was effective *in vitro* against *S. mansoni* adult worms at a concentration of 100–200 μM (Cunha et al., 2012). The triphenylphosphonium derivatives of betulin and betulinic acid showed *in vitro* antischistosomal activity against newly transformed schistosomula and adult worms of *S. mansoni* at concentrations between 10 μM and 2 μM, respectively (Spivak et al., 2014). Moreover, balsaminol F and karavilagenin C are cucurbitane-type triterpenes from *Momordica balsamina* with effective *in vitro* antischistosomal activity against *S. mansoni* adult worms at LC_{50} values of 15 and 29 μM respectively (Ramalhete et al., 2012). Crude extracts and fractions of different families of plants have also indicated antischistosomal activity at varying degrees of potency. The stem bark and roots of *Rauwolfia vomitoria* killed all cercariae stage of *S. mansoni* within 2 h of exposure at a respective concentration range of 62.5–1000 μg/ml and 250–1000 μg/ml. The LC_{50} values after 1 and 2 h was 207.4 μg/ml (stem) and 61.18 μg/ml (root). In addition, both the stem and root of the plant showed 100 mortality of the adult worm within 120 h of incubation at a concentration range of 250–1000 μg/ml (Tekwu et al., 2017). The MeOH extracts of *Curcuma longa* L. (Zingiberaceae) and *Nerium oleander* L. (Apocynaceae) showed 100% *S. mansoni* worm mortality after a 24 h incubation period at concentrations up to 100 μg/ml (Abdel-Hameed et al., 2008). Also, MeOH extracts of five plants *Dryopteris filixmas* (Dryopteridaceae), *Tanacetum vulgare* (Asteraceae), *Juglans nigra* (Juglandaceae), *Syzygium aromaticum* (Myrtaceae) and *Allium sativum* (Liliaceae) exhibited strong potency at minimum effective concentrations of 50 μg/ml after 24 h against adult *S. mansoni* worms (Metwalley, 2015). In a study to evaluate the effect of MeOH extracts on *S. mansoni* infected Swiss Albino Mice, *Malus domestica* (Rosaceae) showed significant ($P < 0.05$) antischistosomal activity at concentrations 300 mg/kg and 200 mg/kg with respective worm reduction of 85.93% and 72.22%. *Allium cepa* (Liliaceae), at 500 mg/kg and 300 mg/kg indicated 72.59% and 58.52% worm reductions respectively, whiles *Citrus limon* (Rutaceae) showed the least worm reduction of 42.96% at 200 mg/kg and 26.63% at 100 mg/kg (Muema et al., 2015). In a similar study, different solvent extracts of *Ocimum americanum* (Lamiaceae) and *Bridelia micrantha* (Phyllanthaceae) gave significant antischistosomal activity against *S. mansoni* infected Swiss Albino Mice (Waiganjo et al., 2016).

4. Conclusion

This study has established the presence of tetracyclic and pentacyclic triterpenoids from the stems and roots of *D. crassifolium*. The identification of dichapetalins from the plant is chemotaxonomically significant since other species of the genus have been shown to contain this unique class of triterpenoids. So far, nine species of this genus have been shown to contain the dichapetalin class of compounds. However, only the roots of *D. crassifolium* contained both dichapetalins A and M, the stem yielded only dichapetalin M. For the first time, maslinic acid and pomolic acid have been isolated from both the stems and roots of the plant. The activity of both extracts and isolated triterpenoids against *S. haematobium* was very low to merit consideration as potential lead compounds for development into any *anti-schistosomal* agent. The extracts and compounds might have an effect on other stages of the diseases such as the schistosomulae, miracidiae and the cercariae. Thus, a study of infections from experimental animals might reveal useful information on this. The general low activity of the tested compounds and extracts may be attributed to the facts that eggs of schistosomes have multiple layers between the shell and the larva. Hence, the presence of these layers, together with the minute size of pores in the egg shells, may preclude penetration of eggs by the extracts used.

Declarations

Author contribution statement

Mary Anti Chama: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Dorcas Osei-Safo: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ivan Addae-Mensah: Conceived and designed the experiments.

Michael Wilson: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Henry Akwaffo Onyame, Claudine Fleischer, Reiner Waibel, Joseph Otchere: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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