Research Article

Anti-onchocercal and antibacterial Activities of crude extracts and Secondary Metabolites from the Rhizome of *Anchomanes difformis* (Araceae)

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ABSTRACT

The methanol (MeOH) extract of *Anchomanes difformis* (Araceae) was fractionated by column chromatography and some pure compounds were obtained whose structures were determined by ^{1}H and ^{13}C-NMR spectroscopy. The extract was tested *in vitro* for anti-onchocercal activity against *O. ochengi,* a close relative of *O. vulvolus*. The MeOH extract showed 100% inhibition of *O. ochengi* microfilarial activity. Compounds **1**, **2** and **3** which were identified as (Z)-12-heptadecenoic acid (**1**), hexadecanoic acid (**2**) and β-stigmasterol (**3**) showed no anti-onchocercal activity against the microfilariae, but **1** and **2** showed weak antibacterial activity against *E. coli* with erythromycin included as reference.The present study constitutes the first report on the antifilarial activity of *Anchomanes difformis* and lends credence to the traditional use of juice from the rhizome in the management of river blindness.

Key words: Anchomanes difformis, Onchocerciasis, Antibacterial, Antifilarial

Résumé

L'extrait méthanol (MeOH) d'*Anchomanes difformis* a été fractionné par chromatographie sur colonne et certaines composés purs ont été obtenues dont les structures ont été déterminées par ^{1}H et ^{13}C-NMR. L'extrait MeOH du rhizome d'*Anchomanes difformis* (Araceae) a été testé *in vitro* pour l'activité contre *O. ochengi*, un proche parent d'*O. vulvolus*. L'extrait MeOH a montré 100 % d'inhibition d'activité microfilarienne *O. ochengi.* Les composés **1**, **2** et **3** qui ont été identifiés comme l'acide (Z) -12-heptadécénoiques (**1**), l'acide hexadécanoïque (**2**) et β-stigmastérol (**3**) ont exhibés aucune activité contre les microfilaires, mais **1** et **2** ont une activité antibactérienne faible contre *E. coli* avec l'érythromycine inclus comme référence. La présente étude constitue le premier rapport sur l'activité antifilariens d'*Anchomanes difformis* et ajoute foi à l'usage traditionnel du jus du rhizome dans la gestion de l'onchocercose.

Mots clés: Anchomanes difformis, Onchocercose, Antibactérienne, Antifilariens

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Introduction

Infectious diseases are a permanent challenge to human health worldwide. Bacterial, viral and protozoal infections are common major causes of mortality (WHO 2014a) which are being tackled using various control and prevention tools. However seventeen infectious diseases are listed as neglected including onchocerciasis (WHO, 2014b).

Onchocerciasis or subcutaneous filariasis, commonly known as river blindness, is one of the 17 neglected tropical diseases (WHO 2014b). It is caused by the filarial worm *Onchocerca volvulus* and transmitted by the black fly, *Simulium damnosum*. The World Health Organization (WHO) report stipulates that 30 of the 37 countries in the world where onchocerciasis is prevalent are in Africa and account for 99% of the global burden of onchocerciasis and onchocerciasis-related maladies. The remaining 1% of cases is confined to Yemen and some countries of Central and South America. In addition, the WHO estimates that over 120 million people worldwide are at risk of infection with onchocerciasis. It is the world's second-leading infectious cause of human blindness with 270,000 of those infected with *Onchocerca volvulus* reported to be blind and 500,000 are suffering from visual impairment (WHO 2010). Overall the disease has a high negative impact on the welfare and economy of the affected populations. Recently, it has been reported that the Onchocerciasis Control Program in West Africa has successfully controlled the disease by large scale vector control in the endemic savanna region of nine West African countries (Zouré *et al.* 2014). In South America, Colombia is the first country in the world to be conferred elimination status for onchocerciasis by the WHO (WHO Fact Sheet update March 2014, No. 374).

In Cameroon, the disease prevalence stands at 31.9% (Boussinesq *et al.* 2002; TDR, 2007; Alemnji *et al.* 2007). About 5.1 million people are at risk and about 1.5 million are already infected mostly in the northern part of Cameroon with some 26,000 people already blind due to onchocerciasis (Albano *et al.* 2010). Mass drug administration (MDA) and vector control programs have for the past several years been the major approaches to control the disease (Wanji *et al.* 2009). Ivermectin (IVM) (Figure 1), which is the main recommended chemotherapeutic agent currently in use, has very limited macrofilaricidal efficacy, and treatment for the life-span of the adult worm (about 18 years) is needed in order to terminate transmission of the parasite .

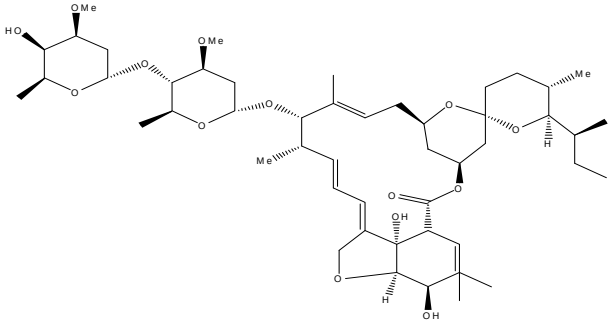

Figure 1. Chemical structure of Ivermectin

This long period of treatment may lead to patients' noncompliance and drug resistance in the parasite. The emergence of ivermectin resistance in parasitic nematodes in veterinary medicine (Lustigman *et al.* 2007) may be an indication that this may extend to the human *O. volvulus*. (Winnen *et al.* 2002, Osei-Atweneboana *et al.* 2007). In addition, it has been revealed that countries where onchocerciasis had been previously eliminated were re-infected and the treatment of potential breeding sites with larvicides had to resume because *S. damnosum* can fly long distances (Baker *et al.* 1990). These challenges coupled with adverse effects of ivermectin to humans (Fay *et al.* 2007) have prompted an urgent search for new and more potent drugs for the treatment of river blindness.

Medicinal plants, which provide chemical scaffolds of most synthetic drugs, have been the

mainstay of African pharmacopeia for centuries and 80% of the inhabitants of this continent depend on medicinal plants for their health problems (WHO, 2008). A review by Ndjonka *et al.* (2013) on natural products as a source for the treatment of neglected tropical diseases, lists some of the plants and a few pure compounds, which have shown anti-onchocercal activities against both micro- and macrofilariasis since 2002. Metuge *et al.* (2014) have reported that the hexane extract and essential oils of roots and rhizomes of *Cyperus articulatus* L. have shown anti-onchocercal activity *in vitro* against both micro- and macrofilariae of *Onchocera ochengi*, a close relative of *Onchocerca volvulus*.

As part of the ongoing effort to search for antifilarial drug leads from medicinal plants of Cameroon, we embarked on the phytochemical study and biological screening of the rhizome of *Anchomanes difformis* (Araceae), a medicinal plant used in traditional medicine by the people of Korup (locally known as "ewomaben") for the treatment of filarial worms (Thomas *et a*l., 1989). The aim of this work was to screen for anti-onchocercal activity of *Anchomanes difformis*, through *in vitro* bioassay-guided testing and to corroborate its use in traditional medicine for the treatment of subcutaneous filariasis and other diseases, and contribute to the pool of drug leads that could be developed into efficacious drugs. A phytochemical analysis of the active fraction was also envisaged.

Anchomanes difformis is an herbaceous plant that grows predominantly in the tropical forest of Africa. It has shown diversity in its biological activities ranging from gastro-protective (Okpo *et al.* 2011), anti-trypanosomal (Atawodi *et al.* 2003), anti-microbial (Eneojo *et al.* 2011), antiplasmodial (Bero *et al.* 2009), insecticidal and toxicological (**A**kinkurolere, 2007) and anticancer (Soladoye *et al.* 2010) effects. Previous work done on the extract of this plant (leaves, tubers/roots and stem barks) showed that it contains saponins, tannins, alkaloids, terpenoids, carbohydrates and cardiac glycosides (Afolayan *et al.* 2012), flavonoids and phenols (Aliyu *et al.* 2008). Given the wide ranging antimicrobial properties of *A. difformis* mentioned above, antibacterial screening of secondary metabolites of this plant was also attempted in this study. This was motivated by the increasing resistance to almost all classes of antibiotics (Davies and Davies, 2010) and the slow turnover in new antibacterials approved for clinical use which are compromising antibiotics chemotherapy (Bassetti *et al*, 2013). Resistance to antibiotics is widespread and has also been reported in the Cameroonian population (Gangoue-Pieboji *et al*, 2006, Ndip *et al*, 2008).

2. Experimental

2.1 Plant materials

The rhizome of *Anchomanes difformis* was harvested in August 2011 in Buea, South West region and identified in collaboration with botanists at Limbe Botanic Garden where a voucher specimen (Ref. No. SCA943) has been deposited.

2.2 Extraction and isolation of Plant Material

Chopped oven-dried (60–70 °C) rhizome of *Anchomanes difformis* was ground into coarse powder. A weight of 6.0 kg was obtained and macerated with MeOH for 3 days x 3 with repeated stirring. The extract was concentrated using a rotary evaporator (Büchi 461 Brinkmann) under reduced pressure to a minimum volume and allowed to stand. A crude extract of 192.0 g was obtained and impregnated with an equal mass of celite. The mixture was subjected to column chromatography with EtOAc/Hex as eluent at varying polarity. The fractions, A, B, C, D, E, and F (as labeled in Table 1 below) obtained were further subjected to column chromatography to obtain compounds **1, 2** and **3.** Compounds **1** and **2** were obtained from fraction E (fraction obtained at 20-60% EtOAc in Hexane) while compound **3** was obtained from

fraction C (fraction obtained at 5-10% EtOAc in Hexane). The main fractions B, C, D and E produced 100% inhibition of microfilarial activity.

Table 1: Flash Chromatographic Profile of the methanol crude extract

Eluent EtOAc in Hexane	Combined fractions (series)	Mass (g) obtained	Remarks
0%	1 – 9 (A)	14.1	Mixture of Compounds
5-10%	10 – 23 (B)	17.4	Mixture of Compounds
5-10%	24 – 29 (C)	2.9	Mixture of Compounds + compound **3** (267 mg)
10-20%	30 – 40 (D)	8.2	Mixture of Compounds
20-60%	41 – 77 (E)	6.1	Mixture of Compounds + (compound **1**, 39 mg and compound **2**, 41 mg)
60-100%	78 – 98 (F)	2.9	Mixture of Compounds

^{1}H and ^{13}C-NMR were carried out on a WALTZ-16 modulated spectrometer using $CDCl_3$ as solvent. The chemical shift values were given in parts per million (ppm) on the δ-scale. The melting points of pure compounds were recorded using a Melt-Temp II apparatus. Column chromatography was carried out using Merck silica gel 60 (particle size 60-200 μm) and Sephadex LH20. For thin layer chromatography (TLC) analysis, pre-coated aluminum sheets with silica gel (Alugram SIL/UV254) were used. Zones on these plates were visualized under UV GL-58 lamp at 254 and/or 365 nm. Alternatively, these plates were exposed to iodine vapour in an iodine chamber.

The structures of the isolated compounds were elucidated by ^{1}H NMR and ^{13}C NMR spectroscopy (1D) and measurement of melting points and the data obtained compared with published data in the literature (Pari *et al.,* 2000, Keat *et al.,* 2010, and Isa *et al.,* 2012) for compounds **1, 2,** and **3** respectively. The spectroscopic data of three fully characterized isolates are as follows: (Z)-6-methyl-12-heptadecenoic acid (**1**): white colour crystals, mp 55-60 °C, ^{13}C NMR (125 MHz, $CDCl_3$) δ(ppm): 179.2 (C-1), 33.4 (C-2), 29.4 (C-3), 24.7 (C-4), 31.9 (C-5), 29.7 (C-6, C-7), 29.5 (C-8), 29.6 (C-9, C-10), 29.1 (C-11), 129.3 (C-12, C-13), 29.2 (C-14), 29.4 (C-15), 22.7 (C-16), 14.1 (C-17).

Hexadecanoic acid (**2**): white colour crystals, mp 39-43 °C, ^{13}C NMR (125 MHz, $CDCl_3$) δ(ppm): 179.2 (C-1), 34.0 (C-2), 24.7 (C-3), 29.0-29.7 (C-4 – C-13), 31.9 (C-14), 22.7 (C-15), 14.1 (C-16).

β-stigmasterol (**3**): white colour crystals, mp 133 °C, ^{13}C NMR (125 MHz, $CDCl_3$) δ(ppm): 37.3 (C-1), 31.7 (C-2), 71.8 (C-3), 42.2 (C-4), 140.8(C-5), 121.7 (C-6), 31.9 (C-7, C-8), 51.2 (C-9), 36.5 (C-10), 21.2 (C-11), 39.8 (C-12), 42.3 (C-13), 56.9 (C-14), 24.4 (C-15), 28.9 (C-16), 56.1 (C-17), 12.2 (C-18), 19.8 (C-19), 40.5 (C-20), 21.1 (C-21), 138.3 (C-22), 129.3 (C-23), 50.2 (C-24), 33.9 (C-25), 23.1 (C-26), 19.4 (C-27), 26.1 (C-28), 12.0 (C-29).

2.3 O. ochengi microfilariae primary screens and cytotoxicity

Microfilariae were extracted by the method of Cho-Ngwa *et al*, 2005 with slight modification. Briefly, *O. ochengi* infected cattle skins obtained from the slaughter house in Buea was washed and sterilized with 70% ethanol. A portion of the skin was shaved, washed in distilled water and sterilized. Criss cross cuts were made on the skin that was firmly attached to a cylindrical wood and the entire skin transferred into a glass cylinder containing complete culture medium (RPMI-1640 supplemented with 25 mM HEPES, 2 g/L sodium bicarbonate, 20 mM L-glutamine, 10% new born calf serum [SIGMA, USA], 200 units/mL penicillin, 200 μg/mL streptomycin and 2.5 μg/mL amphotericin B [Sigma, USA]), pH 7.4). After incubation (for 5 hours), the microfilariae were concentrated by centrifugation quantified by microscopy and cultured on confluent feeder layer of monkey kidney epithelial cells (LLCMK2) in 96 wells culture plates. LLCMK2 cells were purchased from the American Type Culture Collection (ATCC,

Virginia, USA) and proliferated in complete culture medium.

Culture medium was removed from the feeder layer by swift decantation and replenished by 100 µL of fresh complete culture medium containing approximately 15 highly motile microfilariae per well. A stock solution of 5 mg/mL of the compounds were prepared in ≥ 99.9% sterile dimethyl sulfoxide (SIGMA, USA) and tested at 500 µg/mL. After addition of drugs, the microfilariae were cultured at 37°C under an atmosphere of 5% CO_2 in humidified air in a HERACELL-150 CO_2 incubator (Thermo Electron, Germany). Amocarzine (10 µg/mL) was used as the positive control while the negative control wells contained only the culture medium and drug diluent. Worm viability was assessed every 24 hours for a period of five days using an inverted microscope (Nikon TMS, BERGSTROM INSTRUMENT AB). Cytotoxicity was also assessed by microscopy. A compound was considered active if there was 100% inhibition of motility, moderately active if there was 50 – 99% reduction in motility and inactive if there was <50% reduction in motility. With 100% inhibition of motility were considered highly active while those with 25% to 75% were considered moderately active. The assay was repeated to confirm the results obtained.

2.4. In vitro Antibacterial Assay

The bacterial isolates, *Escherichia coli* and *Staphylococcus aureus*, were isolated from clinical specimens from patients consulting in health facilities in Buea. The organisms were cultured on Muller Hinton agar in culture plates prepared according to the manufacturer's instructions, and the cultures stored at 4 °C. The antibacterial screen was performed as described (Mbah *et al.*, 2012) with some modifications. Briefly sterile discs (5-6 mm diameter) were prepared from Whatman filter paper. Five (5) milligrams of each test substance was dissolved in 1 mL of methylene chloride, to give a concentration of 50 µg/10µL. Discs were impregnated with test substance (50 to 100 µg per disc) and the solvent dried-off. A commercial erythromycin disc and a negative control disc containing methylene chloride (prepared similarly) were included as positive and negative control respectively. The Kirby-Bauer (spread plate) method was then performed with the discs gently fixed on a uniform spread of bacterial suspension (approximately 6 x 10^8 colony forming units (CFUs)/mL in 0.85% saline equivalent to McFarland 2) and incubated for 18 hours at 37 °C and the zones of inhibition measured in millimetres. The minimum inhibitory concentration (MIC) of the methanol crude extract from which the fractions and pure compounds were isolated was determined as described (Mbah et al, 2012) using Mueller Hinton broth. A stock of the extract was prepared by dissolving the required amount initially in dimethyl sulfoxide (DMSO) and the volume made up with broth to give 20% v/v DMSO. The extract was incubated in duplicate wells at final concentrations ranging from 0.5 to 10 mg/mL (5% final DMSO concentration) with a bacterial inoculum of 5 x 10^5 CFUs /mL in a microtitre plate. Negative control (no test substance) and gentamycin positive control wells (final concentration of 12 µg/mL) were included. The plate was incubated as above and the MIC taken as the lowest concentration showing no bacterial growth. All experiments were conducted twice.

3. Results

3.1 Identification of Compounds

Compound **1** (Figure 2) was obtained as a white solid with melting point in the range 55-60 °C.

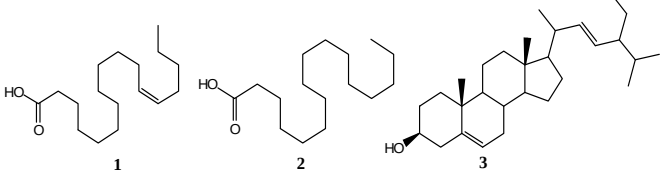

Figure 2: Chemical Structures of compounds **1**, **2** and **3** isolated from the methanol extract of *Anchomanes difformis*

The ^{1}H NMR spectrum shows the characteristics of aliphatic carboxylic acid at region δ0.92 - 2.40. The spectrum indicates the presence of long chain methylene protons representing twelve methylene groups which overlap as a broad peak at δ1.23. There also occur methene protons at δ5.38 and 5.23 (C-13 and C-12 protons respectively). The ^{13}C NMR spectrum shows a terminal methyl carbon C-17 at δ14.2 attached to methylene carbon C-16 (δ22.7), while methylene carbons C-3, C-6 to C-11 and C-14 to C-15 overlap at δ29.1 – 29.7. The existence of carbonyl signal was observed at δ179.2. The DEPT spectrum indicates 17 signals for 1 C, 1 CH_3, 2 CH and 13 CH_2. Based on literature, compound **1** was identified to have a Z-geometry (*cis* stereochemistry). This is supported by the lack of absorption around 960-980 cm^{-1} region in the IR spectrum. It has been reported that allylic carbons of E-geometry of alkenes have δ≥ 30.0, while Z-geometry have δ≈27.0. The chemical shift values of the allylic carbons (C-11, 29.1 and C-14, 29.2) are consistent with Z-configuration (Pari *et al.* 2000). The FT-IR spectrum showed the following bands, 2925 and 2810 cm^{-1} (C-H stretch), 1710 cm^{-1} (C=O stretch), 1290 cm^{-1} (C-O stretch), 1419 cm^{-1} (O-H bend) and 750 cm^{-1} (=C-H bend). Comparison of the experimental data (^{1}H, ^{13}C and FT-IR) with literature values revealed a match with (Z)-6-methyl-12-heptadecenoic acid (except at carbon number 6 bearing the methyl group) a derivative of (Z)-12-heptadecenoic acid previously isolated from the essential oils of *Ageratum conyzoides* (Pari *et al.* 2000). It has a molecular formula of $C_{17}H_{32}O_2$ and two degrees of unsaturation.

Compound **2** (Figure 2) was obtained as a white solid crystal with melting point in the range 39-43 °C, which is in agreement with the value presented in literature 38- 40 °C for hexadecanoic acid (Keat *et al.*, 2010). The ^{1}H-NMR spectrum shows the characteristic of aliphatic carboxylic acid at region δ0.80 - 2.40. The spectrum indicated the presence of long chain methylene protons representing eleven methylene groups which overlapped as a broad peak at δ1.23. The ^{13}C NMR spectrum shows the presence of seven peaks. The terminal methyl carbon C-16 appeared at δ14.1 attached to methylene carbon C-15 (δ 22.7). Another methylene carbon resonates at δ 31.9 (C-14), while methylene carbons C-4 to C-13 overlapped between δ29.1 – 29.7. The existence of carbonyl signal was observed at δ179.9. The DEPT spectrum show 16 signals for 1 CH_3, 14 CH_2, and 1 C. Therefore, with the guidance of the spectra analyses and literature results, we identified compound 2 as hexadecanoic acid; with molecular formula $C_{16}H_{32}O_2$ and one degree of unsaturation.

Compound **3** (Figure 2) was obtained as a white crystal with melting point recorded as 133 °C which is consistent with the value presented in literature for β-stigmasterol (Habib *et al.*, 2007). The ^{1}H-NMR spectrum of AD1 shows peaks for two tertiary methyl groups at δ0.84 and δ0.82 (C-19 and C-18 protons respectively), three multiplets at δ5.34 (C-6 proton), δ5.14 and δ5.04 (C-22 and C-23 protons respectively). The peak at δ3.52 ppm, triplet of a double doublet, was assigned to C-3 proton. The ^{13}C-NMR spectrum shows some recognizable signals at δ140.8 (part of a fused ring) and δ121.7 ppm which is assigned to the sp^2 carbons C-5 and C-6 respectively. According to literature, β-stigmasterol has a β-hydroxyl group attached to C-3 which accounts for the high δ value at 71.8 (Isah *et al.,* 2012). The signals observed at δ12.2 and δ19.4 were assigned to the angular carbon atoms at C-18 and C-19 respectively. The lower δ value for C-18 is attributed to γ-gauche interaction that increases the screening of C-18 thereby causing the lowering of its chemical shift. The DEPT spectrum showed 29 clear signals for 6 CH_3 (two of which were tertiary methyl groups), 9 CH_2, 11 CH (one of which was oxygenated at C-3) and 3 C. Therefore, with the guidance of the spectra

analyses and literature results, we identified compound **3** as β-stigmasterol with molecular formula $C_{29}H_{48}O$ and 6 degrees of unsaturation.

3.2 Anti-onchocercal and antibacterial activities

All bioassays were performed in the Biotechnology Unit of the University of Buea. The methanol crude extract, some main fractions and pure compounds isolated from *A. difformis* were tested *in vitro* on *O. ochengi* and the results are summarised in Tables 2a and b below. The crude extract, main fractions and pure compounds were tested in duplicate wells (200 µL/well) beginning from 500 µg/mL for the crude extracts (plus fractions) and 10 µg/mL for pure compounds The methanol extract and the four main fractions (B, C, D, E) completely inhibited mfs motility at the highest concentration of 500 µg/mL. The active extracts were also cytotoxic Compounds 1, 2 and 3 isolated from main fractions C and E were inactive suggesting a combination effect in the activity of the crude extract and active fractions. Dose-dependent assay of the extract gave an IC_{50} of 250 µg/mL and a selectivity index of 0.5 (Table 2b).

The MIC of the etract was high (> 10 mg/mL) suggesting relatively weak antibacterial activity. However, the antibacterial activity of the fractions ranged from 0 to 15 mm whereas the pure compounds produced zones ranging from 8 to 12 mm. It was also observed that the negative control showed an interference zone of 0 to 10 mm suggesting this solvent likely contributed to the zones produced by the test substances. This was likely an artefact given that this effect was not consistent. The fractions were more active on *E. coli* (8 – 15 mm) than *S. aureus* (0 - 9 mm).

Table 2a: Effect of pure compounds and fractions on motility of *O. ochengi* microfilariae on day 5 of culture

Test substance	**% inhibition at 10 µg/mL**	**Remark on cytotoxicity**	**Remark on activity**
Compound **1**	0	Non-toxic	Inactive
Compound **2**	0	Non-toxic	Inactive
Compound **3**	0	Non-toxic	Inactive
	% inhibition at 500 µg/mL	**Remark on cytotoxicity**	
1 – 9 (A) 100% Hexane	25	Cytotoxic	Inactive
10 – 23 (B) EtOAc in Hexane, 5-10%	100	Cytotoxic	Active
24 – 29 (C) EtOAc in Hexane, 5-10%	100	Cytotoxic	Active
30 – 40 (D) EtOAc in Hexane, 10-20%	100	Cytotoxic	Active
41 – 77 (E) EtOAc in Hexane, 20-60%	100	Cytotoxic	Active
78 – 98 (F) EtOAc in Hexane, 60-100%	0	Non-toxic	Inactive

Table 2b: Dose-dependent inhibition of *O. ochengi* microfilariae by *A. difformis*

Concentration of *A. difformis* (µg/mL)	**% inhibition of mfs motility by day 5**	**Cytotoxicity**	**Remark on activity**
500	100	100% cytotoxic	Active
250	50	100% cytotoxic	Moderately active
125	25	50% cytotoxic	Inactive
62.5	0	Non-toxic	Inactive
31.25	0	Non-toxic	Inactive
15.63	0	Non-toxic	Inactive
7.81	0	Non-toxic	Inactive
0	0	Non-toxic	Inactive

4. Discussion

Three compounds were isolated from the rhizome of *A. difformis* and characterized as two long chain carboxylic acids, (Z)-12-heptadecenoic acid (**1**) and hexadecanoic acid (**2**), and a sterol, β-stigmasterol (**3**).

Table 3: Antibacterial screening of natural products from Anchomanes difformis

Test substance	Composition	*E. coli*	*S. aureus*
		Minimum Inhibitory Concentration (mg/mL)	
Crude extract	Methanol extract	> 10	> 10
Fractions		Zone of inhibition (mm)	
Fraction C	Compound 3	(11-12)*	00
Fraction E	Compounds 1 and 2	(11-15)*	9-11
Positive control	Ciprofloxacin	13, 25*	12,25*
Negative control	Methylene chloride	7-10α	00
Pure compounds†	Compound 1	10-11	-
	Compound 2	10-12	-
	Compound 3	8-9	-
Positive control	Erythromycin	25	-
Negative control	Methylene chloride	9-10α	-

* Indicates clear and unclear zone areas: the first figure represents a clear inner area (no bacterial colonies) closer to the disc and the second digit the total zone including an outer unclear part (with scanty bacterial colonies). Zones in parentheses are totally unclear (scanty colonies present all over the zone).

α Interference of negative control (CH_2Cl_2) in assay.

† Pure compounds at 100µg per disc

– Not done

Though none of the pure compounds showed any activity against *O. Ochengi*, the crude MeOH extract of *A. difformis* showed 100% inhibition of mfs activity. This result, to the best of our knowledge, establishes for the first time, the antifilarial activity of the plant and lends credence to its traditional use in the treatment of river blindness. The plant and other related species should, therefore, be evaluated for antifilarial activity. A derivative of compound **1**, (Z)-6-methyl-12-heptadecenoic acid, isolated from *Ageratum conyzoides*, has been shown to possess insecticidal and growth regulatory activity against the desert locust *Schistocerca gregaria*. This can therefore explain the insecticidal properties of *A. difformis* (Akinkurolere, 2007). Meanwhile two similar studies have demonstrated the antifilarial potential of plants used in traditional medicine to treat onchocerciasis. Cho-Ngwa *et al*. (2010) reported similar results with 100 % inhibitory activity of crude extracts of two plants, *Margaritaria discoidea* [Baill] Webster (Euphorbiaceae) and *Homalium africanum* [Hook. f] Benth (Flacourtiaceae), against *O. ochengi* microfilariae at 60 to 500 µg/mL. Another study (Metuge *et al.*, reported considerable activity (15 to 45 µg/mL) of two fully characterised compounds isolated from *Cyperus articulatus* (Cyperaceae). The findings of these studies are evidence of the activity of natural products against onchocerchaisis and suggest the activity observed for the methanol crude extract in this study may be a combined effect of the secondary metabolites present in it but this remains to be investigated in a further experiment.

Both the fractions and pure compounds showed antibacterial activity against the Gram –negative *E. coli* and Gram-positive *S. aureus*. This confirms the earlier reports on the antibacterial potential of *A. difformis*.

Although the active extracts were also cytotoxic on LLCMK2 cells, it would appear that the plant is safe in individuals who use the concoction for filarial treatment. Findings by Cho-Ngwa *et al.* (2010) and Samje *et al.* (2014) on cytotoxic extracts tested in mice showed that cytotoxicity does not necessarily imply toxicity in *in vivo* systems. When compared with ivermectin in the same assay, the cytotoxic extract had a higher selectivity index than ivermectin. A detoxification mechanism might account for the safe toleration of the extract in the human system. It is therefore important that following cytotoxicity studies, acute toxicity studies should also be conducted to better ascertain the safety profile of the extract/ compound.

5. Conclusion

Though an active pure compound against river blindness was not obtained in this study, these results provide some support for the traditional use of the plant for management of river blindness and the plant is thus identified as a potential source of antifilarial lead compounds. On the basis of the quantities of test substance used, the zones of the positive controls and the zones produced by the test substances, the antibacterial activity of the test substances is relatively weak compared to standard antibiotics..

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References

Afolayan, M. O., Omojola, M. O., Onwualu, A. P., Thomas, S A. (2012): Further physicochemical characterization of *Anchomanes difformis* starch. *Agriculture and Biology Journal of North America*, ISSN Print: 2151-7517, ISSN Online: 2151-7525]

Akinkurolere, R. O., **(**2007**):** Assessment of the Insecticidal Properties of Anchomanes difformis Powder on Five Beetles of Stored Produce. *J. of Entomology* 4 (1), 51-55

Albano, P., Barbell P., Bengraine, K., Brown, N., Dimar, A., Eckert, K., Friedman, J., Prendki, G. (2010**):** Lion's Onchocerciasis Control efforts in Cameroon. Lions Club International Foundation, pp 1-7.

Alemnji, G. A., Mbuagbaw, J., Toukam, K.E., Ayong, L., Wembe, E., Asonganyi, T. (2007**):** High prevalence of co-infection of Human Immune Virus-1 (HIV-1) and onchocerciasis in Cameroon. *Journal of Med Science* **7** (5), 897-900.

Aliyu, A.B., Musa, A.M., Oshanimi, J.A., Ibrahim, H.A., Oyewale, A.O., (2008**)**: Phytochemical Analysis and Mineral Elements Composition of Some Medicinal Plants of Northern Nigeria. *Nigerian Journal of Pharmaceutical Sciences* **7** (1), ISSN: 0189-823X.

Atawodi, S.E., Bulus, T., Ibrahim, S., Ameh, D.A., Nok, A.T., Mamman, M., Galadima, M. (2003**):** In vitro trypanocidal effect of methanolic extract of some Nigerian savannah plants (abstract). *Afr. J. Biotech.* **2** (9), 317.

Baker R. H.A., Guillet, P., Seketeli, P., Boakye, D., Wilson, M. D., Bissan, Y., Garms, R. A., Cheke, R., Sachs, J. B., Davies, M. A., Howe, M. J., Millest, A. L. (1990): Progress in controlling the reinvasion of windborne vectors into the western area of the Onchocerciasis Control Programme in West Africa, *Phil Trans of Royal Society of London* B 328, 731-750.

Bassetti, M., Merelli, M., Temperoni, C. and Astilean, A. (2013): New antibiotics for bad bugs: where are we? *Ann Clin Microbiol Antimicrob* , **12**:22.

Bero, J., Ganfon, H., Jonville, M.-C., Frédérichb, M., Gbaguidi, F., DeMol, P., Moudachirouc, M., Quetin-Leclercq, J., (2009**):** In vitro antiplasmodial activity of plants used in Benin in traditional medicine to treat malaria. *Journal of Ethnopharmacology* **122**, 439–444

Boussinesq, M., Pion S.D., Demanga-Ngangueand Kamgno J. (2002**).** Relationship between onchocerciasis and epilepsy: a matched case-control study in the Mbam Valley, Republic of Cameroon. *Transactions of the Royal Society of Medicine and Hygiene,* **96**, 537–541.

Cho-Ngwa F, Daggfeldt A, Titanji VPK, Gronvik K: (2005). Preparation and characterisation of specific monoclonal antibodies for the detection of adult worm infections in onchocerciasis. *Hybridoma,* 24:283-290.

Cho-N gwa, F., Abongwa, M., Ngemenya, M.N.,Nyongbela, K.D: (2010). Selective activity of extracts of *Margaritaria discoidea* and *Homalium africanum* on *Onchocerca ochengi*. BMC Complementary and Alternative Medicine, 10:62 http://www.biomedcentral.com/1472-6882/10/62.

Davies, J. and Davies, D. (2010): Origins and Evolution of Antibiotic Resistance. *Microbiol Mol Biol Rev*, 74: 417–433 DOI:10.1128/MMBR.00016-10.

Eneojo, A.S., Egwari, L.O., Mosaku, T.O., (2011**)**: *In vitro* Antimicrobial Screening on *Anchomanes difformis* (Blume) Engl. Leaves and Rhizomes Against Selected Pathogens of Public Health Importance. *Advances in Biological Research* **5** (4), 221-225,].

Fay, M.P., Huanga C., and Twum-Danso, N. A. Y. (2007**):** Monitoring rare serious adverse events from a new treatment and testing for a difference from historical controls. *Clinical Trials*, **4**, 598–610.

Gangoue-Pieboji, J., Koulla-Shiro, S., Ngassam, P,, Adiogo, D. and Ndumbe, P. (2006): Antimicrobial activity against gram negative bacilli from Yaounde Central Hospital, Cameroon. *African Health Sciences* 6 (4), 232 – 235.

Habib M.R., Nikkon F., Rahman M., Haque M.E. and Karim M.R.; (2007). Isolation of Stigmasterol and â-Sistoterol from Methanolic Extract of Root Bark of *Calotropis gigantean* (Linn), *Pakistan J. of Biological Sciences*, **10** (22): 4174-4176.

Isah Y., Ndukwe I.G., Amupitan J.O.; (2012). Isolation of Stigmasterol from Aerial Plant Part of *Spillanthes Acmella Murr*, *World J. Life Sci. and Medical Research*; **2** (2):77.

Keat N.B., Umar R.U., Lajis N.H., Chen T.Y. *(*2010). Chemical Constituents from two Weed species of S*permacoce* (rubiaceae), *The Malaysian Journal of Analytical Sciences,* **14** (1 6) - 116.

Lustigman, S., McCarter, J. P. (2007**):** Ivermectin Resistance in Onchocerca volvulus: Toward a Genetic Basis. PLoS *Negl Trop Dis,* 1:e76.

Metuge, J. A., Nyongbela, K. D., Fotso, G., Mbah, J. A., Samje M., Babiaka, S. B., Cho-Ngwa, F. (2014): Anti-Onchocerca activity and phytochemical analysis of an essential oil from *Cyperus articulatus* **L.** BMC Complementary and Alternative Medicine 14, 223-232.

Metuge, J.A., Babiaka, S.B., Mbah, J.A., Ntie-Kang F., Ayimele, G.A., Cho-Ngwa, F: (2014). Anti-onchocerca Metabolites from Cyperus articulatus:Isolation, In Vitro Activity and In Silico 'Drug-Likeness' *Nat. Prod. Bioprospect*. DOI 10.1007/s13659-014-0023-5.

Ndip, R.N., Ntiege, E.A., Ndip, L.M., Nkwelang, G., Akoachere, J-F. T.K. and Nkuo Akenji, T. (2008): Antimicrobial Resistance of Bacterial Agents of the Upper Respiratory Tract of School Children in Buea, Cameroon. *J Health Popul Nutr* , **26**(4): 397-404.

Ndjonka, D., Rapado, L. N., Silber, A. M., Liebau, E., Wrenger, C. (2013**):** Natural Products as a Source for Treating Neglected Parasitic Diseases. *Int. J. Mol. Sci.*, 14, 3395-3439; doi:10.3390/ijms14023395.

Okpo, S. O., Ching, F. P., Ayinde, B.A., Udi ,O. O., Alonge, P.O., Eze, G.O., **(**2011**):** Gastroprotective Effects of the Ethyl Acetate Fraction of Anchomanes difformis. *International Journal of Health Research* **4** (4), 155-161.

Osei-Atweneboana, M.Y., Eng, J.K., Boakye, D.A., Gyapong, J.O., Prichard, R.K. (2007**):** Prevalence and intensity of *Onchocerca volvulus* infection

and efficacy of ivermectin in endemic communities in Ghana: A two-phase epidemiological study. *Lancet 369*, 2021–2029.

Pari, K., Subrahmanyam, B., Rastogi, J.N., Devakumar, C., Rao, P.J., (2000): Insecticidal (Z)-6-methyl-12-heptadecenoic acid from essential oil of *Ageratum conyzoides*. Indian Journal of Chemistry. **39B,** 451 – 454.

Samje M., Metuge J. A., Mbah J. A., Nguesson B., Cho-Ngwa F. (2014). In vitro anti-Onchocerca ochengi activities of extracts and chromatographic fractions of *Craterispermum laurinum* and *Morinda lucida*. BMC *Complementary and Alternative Medicine*, 14:325.

Soladoye, M. O., Amusa, N. A., Raji-Esan, S. O., Chukwuma, E. C., Taiwo, A. A. (2010**):** Ethnobotanical Survey of Anti-Cancer Plants in Ogun State, *Nigeria. Annals of Biological Research*, **1** (4), 261-273.

TDR (Tropical Disease Research) (2007**):** A new drug for river blindness? *TDR News* **79**, 15-20.

Thomas, D. W., Thomas, J. M., Bromley, W. A., Mbenkum, F. T. Korup Ethnobotany Survey**.** Final Report to the World Wide Fund for Nature, July 1989.

Wanji, S., Tendongfor N., Nji T., Esum, M., Che, J.N., Nkwescheu, A., Alassa, F., Kamnang, G., Enyong, P. Oussinesq, A., Taylor, M. J., Hoerauf, A., Taylor, D. W., **(**2009**):** Community-directed delivery of doxycycline for the treatment of onchocerciasis in areas of co-endemicity with loiasis in Cameroon. *Parasites & Vectors* **2** (39), doi: 10.1186/1756-3305-2-39.

WHO (2010). First WHO report on neglected tropical diseases. Working to overcome the global impact of neglected tropical diseases.

WHO (World Health Organization), (2014): Fact sheet No 310: *The top ten causes of death.*

WHO (World Health Organization), (2014) Fact sheets No 374: Onchocerciasis.

WHO (The World Health Organization), (2008) Facts sheet N°134: Traditional medicine.

Winnen, M., Plaisier, A.P., Alley, E.S., Nagelkerke, N.J., van Oortmarssen, G., Boatin, B.A., Habbema, J.D. (2002**):** Can ivermectin mass treatments eliminate onchocerciasis in Africa? Bull World Health Organ. *80*, 384–391.

World Health Organization: Onchocerciasis and its Control. Report of a WHO Expert Committee on Onchocerciasis Control. Geneva: World Health Organization; 1995:1–104.

Zouré, H. G. M., Noma, M., Tekle, A. H., Amazigo, U. V., Diggle, P. J., Giorgi, E., Remme, J. H. F. (2014): The geographic distribution of onchocerciasis in the 20 participating countries of the African Programme for Onchocerciasis Control: (2) pre-control endemicity levels and estimated number infected. *Parasites & Vectors* 7, 326-340.

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