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Isolation and characterization of filaricidal compounds from the stem bark of *Voacanga africana*, a plant used in the traditional treatment of onchocerciasis in Cameroon

Smith Babiaka Borakaeyabe[1], James Ajeck Mbah[1,3*], Fidelis Cho-Ngwa[2,3], Jonathan Alunge Metuge[2,3] and Simon Mbua Ngale Efange[1,3]

[1]Department of Chemistry, Faculty of Science, University of Buea, Box 63-Buea, Cameroon.
[2]ANDI Centre of Excellence for Onchocerciasis Drug Research, Faculty of Science, University of Buea, Box 63 Buea, Cameroon.
[3]Department of Biochemistry and Molecular Biology, Faculty of Science, University of Buea, Box 63-Buea, Cameroon.

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This study was carried out to isolate active ingredients from *Voacanga africana* Stapf (Apocynaceae) used as herbal medicine in Cameroon, and also to assess the efficacy of the compounds on *Onchocerca ochengi* and *Loa loa* worms. The compounds were isolated using solvent partitioning, column chromatography and fractional crystallization. The *O. ochengi* worms were isolated from cow skin while *Loa loa* was isolated from humans. Filaricidal activity was determined based on motility for adult male worms and microfilariae (Mfs), while adult female worm viability was assessed biochemically by the dimethylthiazol (MTT)/formazan assay. Cytotoxicity was assessed using monkey kidney epithelial cells. Auranofin was used as the positive control drug. Two compounds, voacangine (compound 1) and voacamine (compound 2) were isolated from the stem bark of *Voacanga africana*. Both compounds were found to inhibit the motility of both the microfilariae (Mfs) and adult male worms of *O. ochengi* in a concentration-dependent manner, but were only moderately active on the adult female worms upon biochemical assessment at 30 µM drug concentration. The IC_{50}s for voacangine were 5.49 µM for Mfs and 9.07 µM for adult male worms; while for voacamine the values were 2.49 µM for Mfs and 3.45 µM for adult males. At 10 µM, voacamine showed 100% inhibition of *Loa loa* Mfs motility after 24 h. This is the first report of the anti-*Onchocerca* activity of voacangine (compound 1) and voacamine (compound 2) as well as activity of voacamine (compound 2) on *L. loa*. The results of this study support the traditional use of *V. africana* in the treatment of human onchocerciasis.

Key words: *Voacanga africana*, voacangine, voacamine, anti-*Onchocerca* activity.

INTRODUCTION

Onchocerciasis or river blindness is the second leading infectious cause of blindness in humans globally. According to the World Health Organization, an estimated 37 million people are infected with the causative parasite *Onchocerca volvulus* of which about 300,000 are blind (WHO, 2007). Onchocerciasis also often leads to intense

itching and serious dermatitis. The burden of the disease includes long term disability, social stigmatization and abandonment of the infested areas, which often have a high agricultural potential (Wogu and Okaka, 2008), leading to economic loss and slow down of country development over the years. Over 99% of those infected live in Africa. Rational drug discovery approaches have made only limited advances in the discovery of a safe macrofilaricide against the *Onchocerca* worm.

Ivermectin is the only drug recommended for the treatment of onchocerciasis. Ivermectin was shown to be both safe and effective in the treatment of onchocerciasis, and has become the drug of choice for mass distribution (Gardon et al., 1997). However, ivermectin is only effective against microfilariae (Mfs) and has only limited effects on the adult worm. This, added to mounting evidence of Mfs resistance to ivermectin which made the search for a drug that kills the adult worm (a cure) a research priority area (Lizotte-Waniewski et al., 2000). Unfortunately, pharmaceutical companies have not given a priority to the discovery of anti-filarial drugs because the markets are not incentivizing enough. Indigenous rural communities in the tropics manage parasitic diseases, like onchocerciasis using herbal medicines (Ndjonka et al., 2011).

The efficacy, dosage, safety and active principles of most of the herbal preparations are not known. This study approach has been to investigate medicinal plants that are used for the treatment of onchocerciasis as new sources for filaricides. The study reports the isolation of voacangine (compound 1) and voacamine (compound 2) from *Voacanga africana* and their activities against the *Onchocerca ochengi* worm, the closest known relative of the human parasite, *O. volvulus*. *Voacanga africana* is also used in the management of malaria in Africa (Ameyaw and Duker-Eshun, 2009).

MATERIALS AND METHODS

General

Melting points (mp) were determined on a Meltemp II apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker ARX- 500 spectrometer. The ^{1}H and ^{13}C NMR chemical shifts are expressed in ppm relative to tetramethylsilane (TMS). Thin-layer chromatography (TLC) was performed on Merck silica gel plates. TLC plates were visualized with a UV-lamp (UVGL-58) at 254 or 366 nm and later exposed to iodine. Column chromatography was performed with glass column (60 cm length, 11 cm external diameter) using silica gel 60 to 200 µm.

Plant material

The stem bark of *V. africana* Stapf (Apocynaceae) was collected in Ndop, North West Region of Cameroon, in October, 2011 by Dr. Wirmum Clare, Director of the Medicinal Foods and Plants, Bamenda. A voucher specimen of the plant, N° SCA887 is deposited at the Limbe Botanic Garden.

Extraction and isolation of compounds

Powdered sample of *V. africana* (2 kg) was macerated in 5 L of methanol (MeOH) (3×3 days each), filtered and concentrated on a rotavapor to dryness. A portion of the methanol extract (80 g) was dissolved in 100 ml of methanol and heated at 60°C. The hot solution was poured into an aluminum pot of 300 ml of water while stirring. This aqueous solution (in portions of 100 ml) was then extracted thrice (in a separatory funnel) with methylene chloride (150 ml each). The resulting extract (50 g) was fixed with 70 g of Celite loaded on a column with 80 g of silica gel, and eluted with gradients of ethyl acetate (EtOAc) in hexane. Fractions of ~100 ml were collected, evaporated, recovered with methylene chloride and combined into six groups on the basis of their TLC profiles as shown in Figure 1.

The fractions obtained with 5 and10% ethyl acetate (EtOAc), containing compound 1 were combined into three groups. The fractions 15 to 30, made up the first group (did not contain the non-alkaloid), was evaporated and crystallized from methanol (1.2 g solid). The solid was dissolved in CH_2Cl_2 (5 ml) and methanol (15 ml) was added, then heated to remove CH_2Cl_2 and allowed to crystallize from the methanol at room temperature (0.6 g of compound 1 were obtained). The second group of chromatographic fractions which contained the non-alkaloid component was crystallized from methanol to provide a solid (2 g) which was suspended in hexane with agitation. Upon settling, the non-alkaloid component was found floating at the top while the material at the bottom was enriched in voacangine. After removal of the non-alkaloid component by decantation, the procedure was repeated twice to yield 0.3 g of compound 1. The third group which contained two other alkaloids was treated as the first group to provide 0.2 g of compound 1. The combined mother liquors from the three groups above (3 g) were dissolved in CH_2Cl_2 (10 ml) and extracted with 10% acetic acid (3×100 ml portions). The aqueous phase was neutralized with NH_4OH and extracted with CH_2Cl_2. Crystallization from methanol as with group 1 above afforded 0.3 g of compound 1, *Rf*. = 0.5 (TLC: 15% EtOAc in hexane). The fifth group (obtained with 30 to 60% EtOAc in hexane) was crystallized from MeOH, yielding 10 g of impure compound 2. Purification was done by dissolving the latter in 20 ml of CH_2Cl_2 and adding 50 ml of MeOH, then heating at 40°C to remove CH_2Cl_2, and allowing the material to crystallize yielding 8 g of compound 2, *Rf*. = 0.5 (TLC: 50% EtOAc in hexane).

Compound 1: Glass-like needles, mp 137 to 138°C, ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.90 (3H, t, H-18), 1.12 (1H, m, H-15α), 1.32(1H, m, H-20), 1.44 (1H, m, H-19α), 1.55 (1H, m, H-19β),1.73 (1H, m, H-15β), 1.87 (1H, m, H-14), 1.90 (1H, m, H-17α), 2.57 (1H, ddd, H-17β), 2.80 (1H, d, H-3α), 2.90 (1H, m, H-3β), 2.98 (1H, m, H-5α), 3.13 (1H, m, H-5β), 3.22 (1H, m, H-6α), 3.37 (1H, m, H-6β), 3.54 (1H, brs, H-21), 3.71 (3H, s, CO_2Me), 3.85 (3H, s, OMe), 6.80 (1H, dd, H-11), 6.91 (1H, d, H-9), 7.13 (1H, d, H-12), 7.87 (1H, brs, N-H). ^{13}C NMR ($CDCl_3$, 125 MHz), δ 11.5 (CH_3, C-18), 22.0 (CH_2, C-6), 26.5 (CH_2, C-19), 27.1 (CH, C-14), 31.8 (CH_2, C-15), 36.3 (CH_2, C-17), 38.9 (CH, C-20), 51.3 (CH_2, C-3), 52.4 (CH_3, CO_2Me), 53.0 (CH_2, C-5), 55.1 (C, C-16), 55.8 (CH_3, OMe-10), 57.3 (CH, C-21), 100.5 (CH, C-9), 110.0 (C, C-7), 110.9 (CH, C-12),

*Corresponding author. E-mail: mbah_james@yahoo.com

111.6 (CH, C-11), 129.0 (C, C-8), 130.3 (C, C-13), 137.3 (C, C-2), 153.8 (C, C-10), 175.5 (CO_2Me).

Compound 2: White powder, mp 223°C, ^{1}H NMR ($CDCl_3$, 500 MHz): δ 0.87 (3H, t, H-18), 1.07 (1H, H-15β): δ 0.87 (3H, t, H-18), 1.07 (1H, H-, 1.65 (3H, d, H-18 α),1.78 (1H, H-17 β), 1.80 (1H, m, H-14), 1.98 (1H, H-17 β), 2.46 (3H, s,MeO-22'), 2.48 (1H, H-17 α), 2.57 (1H, H-17'α), 2.58 (3H, s, Me-4'), 2.70 (1H, m, H-3'), 2.72 (1H, H-3α, H–21β), 2.85 (H, H-3β), 2.97 (H, H-6β), 3.09 (H, H-6α), 3.20 (H, H-5α), 3.23 (H, H-6'β), 3.36 (1H, H-5β), 3.48 (1H, H-6'α), 3.50 (1H, s, H-21α), 3.64 (3H, s, MeO-22), 3.78 (1H, m, H-14'), 3.99 (3H, brs, MeO-10), 4.05 (1H,m, H-5'), 5.15 (1H, brd, H-16'), 5.31 (1H, q, H-19'), 6.74 (1H, brs, H-12), 6.93 (1H, s, H-9), 7.04 (1H, t, H-12'), 7.05 (1H, t, H-11'), 7.06 (1H, t, H-10'), 7.49 (1H, brs, HN-1), 7.56 (1H, d, H-9')7.73 (1H, brs, HN-1'). ^{13}C NMR ($CDCl_3$, 125 MHz), δ 11.6 (CH_3, C-18), 12.3 (CH_3, C-18'), 19.5(CH_2, C-6'), 22.2 (CH_2, C-6), 26.7 (CH_2, C-19), 27.3 (CH, C-14), 32.0 (CH_2, C-15), 33.6 (CH, C-14'), 36.4 (CH_2, C-17), 36.4 (CH_2, C-17'), 37.4 (CH, C-16'), 39.0 (CH, C-20), 42.4 (CH_3, Me-4'), 47.0 (CH, C-3'), 49.9 (CH_3, MeO-22'), 52.0 (CH_2, C-3), 52.4 (CH_2, C-21'), 52.4 (CH_3, MeO-22), 53.1 (CH_2, C-5), 54.9 (C, C-16), 56.1 (CH_3, MeO-10), 57.0 (CH, C-21), 59.9 (CH, C-5'), 99.2 (CH, C-9), 109.8 (CH, C-12'), 109.9 (C, C-7), 110.4 (CH, C-12), 117.4 (CH, C-9'), 118.5 (CH, C-19'), 118.8 (CH, C-10'), 121.5 (CH, C-11'), 127.3 (C, C-8), 129.8 (C, C-11), 129.9 (C, C-8'), 130.3 (C, C-13), 135.8 (C, C-13'), 137.1 (C, C-2)137.9 (C, C-2'), 150.9 (C, C-10), 171.6 (C, C-22'), 175.3 (C, C-22).

Isolation of *O. ochengi* adult worms

The isolation of *O. ochengi* adult worms was done as described earlier (Cho-Ngwa et al., 2005). The duration from the slaughtering of a cow to the harvesting of parasites from the skin was always less than 2 h to avoid bacterial growth and reduction in worm viability. Briefly, fresh pieces of umbilical cattle skin with palpable nodules bought from local slaughterhouses were washed, drained and sterilized with 70% ethanol. *O. ochengi* adult worms were carefully scraped out of the nodules as single masses and temporarily submerged in 1 ml complete culture medium (CCM) [RPMI-1640 (SIGMA, USA)] supplemented with 25 mM HEPES, 2 g/l sodium bicarbonate, 2 mM L-glutamine, 5% new born calf serum (SIGMA, USA), 150 units/ml penicillin, 150 µg/ml streptomycin and 0.25 µg/ml amphotericin B [(SIGMA, USA), pH 7.4)] using 24-well plates. The adult worms were allowed in the culture medium overnight in a CO_2 incubator, during which the male worms migrated out of the nodular masses. Only wells containing viable worms received treatment with the isolated test compound. Damaged worms and worms from putrefied nodules were discarded. The viability of worms retained for the assay was ascertained by visual and microscopic examination of adult worm and microfilarial motility using an inverted microscope.

Isolation of *O. ochengi* microfilariae

The cattle skin was obtained as described for adult worms. About 5 skin snips were obtained from different locations of the skin, and incubated separately in small amounts of complete culture medium (CCM) for 30 min. Emerged Mfs were qualified and quantified for *O. ochengi* species with the aid of an inverted microscope. A selected piece of skin, rich in *O. ochengi* Mfs was carefully shaved with a razor blade, and then rinsed with distilled water. It was dabbed with a clean tea cloth to eliminate excess moisture and covered entirely with 70% ethanol. The latter was allowed to evaporate completely in a horizontal flow sterile hood. The ethanol treatment was repeated once. The sterilized skin was tautly attached onto an autoclaved, cylindrical piece of wood using autoclaved thumb nails and close (about 1 mm apart), criss-cross cuts were made into the epidermis and dermis. The assembly was incubated in the culture medium for 4 h. The emergent and highly motile *O. ochengi* microfilariae were concentrated by centrifugation at 400 × g for 10 min and then quantified by counting the Mfs in three 10 µl portions of the Mfs preparation.

Preparation of mammalian cells

Monkey kidney epithelial cells (LLC-MK2) (ATCC, USA) were cultured at 37°C in humidified air with 5% CO_2 in a HeraCell-150 incubator (Thermo Electron, Germany) until the cell layer was almost confluent. The cells were rinsed with a solution of 0.125% trypsin and 0.5 mM edetic acid (EDTA) in medium 199 (Sigma, USA), and kept in the same mixture for less than 1 h for them to be dislodged. The cell suspension was centrifuged at 560 × g for 10 min, the supernatant discarded and the pellet re-suspended to 2 × 10^5 cells/ml in CCM. The cell suspension was dispensed into 96-well microtitre plates (200 µL/ well) and kept in the incubator for 3 to 5 days for cells to grow, and become fully confluent. These cells served as feeder layer for the Mfs assays and were also used for cytotoxicity studies.

Preparation of stock solutions of test compound

Five millimolar (5 mM) stock solution of each compound was prepared by dissolving in 99.9% pure dimethyl sulfoxide (DMSO) (SIGMA, USA). Complete dissolution was achieved by vortexing. The solutions were stored at -20°C before they were used in the assays.

Anti-filarial screening of isolated compounds

Primary screens on adult worms

This was done to eliminate inactive compounds. Adult worm assays were conducted in 24-well plates (NUNC, USA) at 37°C in humidified air containing 5% CO_2 for 5 days (120 h) without change of medium. Nodular masses (each generally containing a few males and a female worm) were first put in the wells (with 1 ml CCM) without test compound overnight to confirm their viability, and for adult males to migrate from the nodule into the culture medium. 12 µl of 5mM stock test compound mixed with 988 µl of CCM was then added into each of quadruplicate wells to give a single final concentration of 30 µM. Four nodular masses each, were used in the negative control (2% dimethyl sulfoxide in CCM only) and in the positive control (10 µM auranofin, a gold conjugated compound) (Bulman et al., 2015) wells in which each well also received only one nodular mass. After 5 days incubation, adult male viability was assessed based on motility scores using an inverted microscope. Motility score was on a scale of 4 (vigorous or normal movement of whole worm, corresponding to 0% inhibition of worm motility), 3 (near normal movement of whole worm or 25% inhibition of worm motility), 2 (whole body of worm motile but sluggish that is, 50% inhibition of worm motility), 1 (only head or tail of worm moving that is, 75% inhibition of worm motility), 0 (completely immotile worm that is, 100% inhibition of worm motility). A compound was considered active if there was a 100% inhibition of adult male worm motility; or moderately active for a motility inhibition of 50 to 99%; and inactive if the inhibition was less than 50%. Adult female worm viability was assessed by the standard colorimetric Dimethylthiazol (MTT)/formazan assay (Comley et al., 1989) in which each nodular mass was placed in a well of a 48 -well microtitre plate containing500 µl/well of 0.5 mg/ml MTT (Sigma, USA) in incomplete

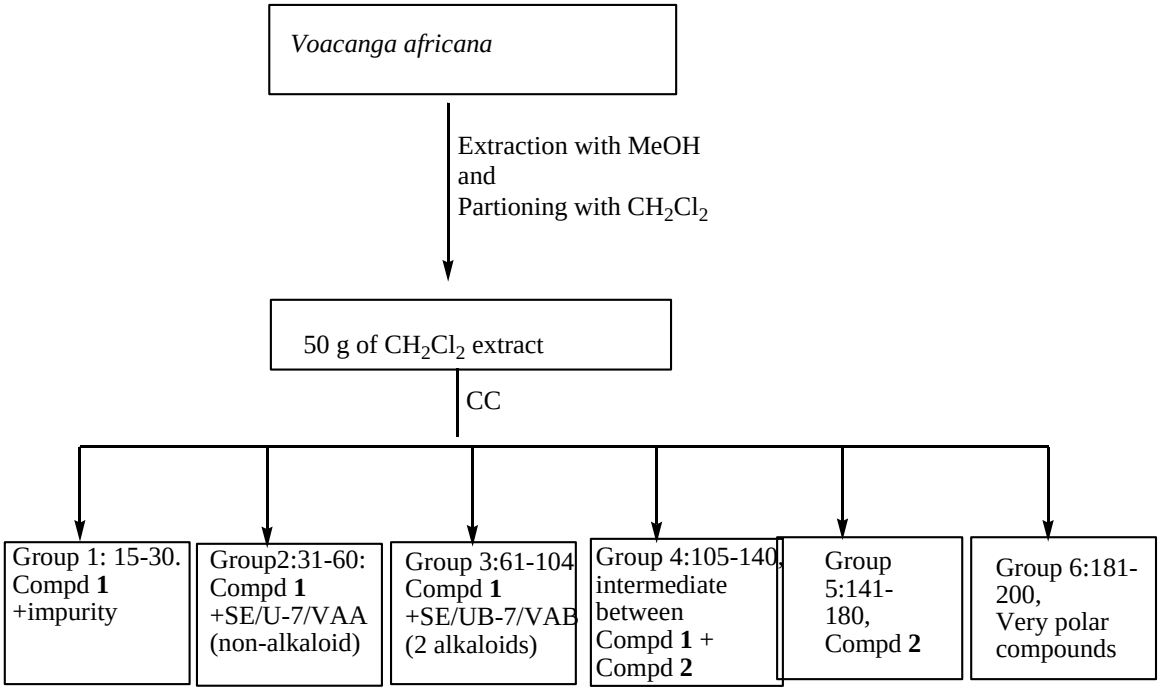

Figure 1. Scheme for the isolation of compounds 1 and 2. SE/UB-7/VAA = non-alkaloid (crystallizes with compd **1**), SE/UB-7/VAB = mixture of 2 alkaloids (appear below voacangine).

Roswell Park Memorial Institute (RPMI) culture medium, and then incubated in the dark at 37°C for 30 min. The degree of colouration of the worms was assessed visually with the help of a digital camera and then by colorimetry. Each nodular mass was placed in 500 µl of Dimethyl sulfoxide (DMSO) in a 48-well plate to allow the colour to elute from the worm for 1 h. After shaking the plate, 200 µL of the coloured formazan solution from each well was pipetted into the wells of a microtitre plate (78 wells), and the optical density read at 490 nm. Adult female worm viability was taken as mean % inhibition of formazan formation relative to negative control at 120 h post addition of the test compound. A compound was considered active on the adult female worm if there was a 90% or greater inhibition of formazan formation compared to the negative controls; or moderately active if the inhibition was 50 to 89%. It was considered inactive if the inhibition was less than 50%. Adult worm death positively correlates with inhibition of formazan formation.

% Inhibition of formazan formation =

$$\frac{\text{OD of negative control} - \text{OD of treatment}}{\text{OD of negative control}} \times 100$$

Primary screen on microfilariae (Mfs)

The compounds were also tested on Mfs at a single concentration of 30 µM, in duplicate wells. The Mfs assay was conducted in 96-well microtitre plates (15 Mfs in 200 µl CCM per well) at 37°C in humidified air containing 5% CO_2 for 5 days without any change of medium. Fully confluent monkey kidney epithelial cells, serving as feeder layer, were co-cultured with the Mfs. The medium used in preparing the feeder cell layer was removed by a swift decantation before fresh CCM containing test compound which was immediately added. Ivermectin (20 µg/ml) and 2% DMSO served as the positive and negative controls respectively. Mfs motility inhibition scores (viability) were done on a scale of 100% (immotile), through 75% (only tail or head shaking occasionally), through 50% (whole body shaking occasionally or moving sluggishly), to 25% (whole body shaking occasionally then to 0% (whole body moving or shaking vigorously). Scores were made every 24 h, terminating at 120 h using an inverted microscope. Any culture with microbial contamination was not considered. Mfs viability was taken as the mean % reduction at 120 h (day 5) after addition of drug. A compound was considered active if there was a 100% reduction in Mfs motility; or moderately active for a motility reduction of 50 to 99%; and inactive if the reduction was less than 50%.

Secondary screens on microfilariae and adult worms

This was done to confirm the activity of the compound after primary screen, and to determine their IC_{50}, IC_{100} and selectivity index (SI) values. The compound was retested as described under primary screens at serial dilutions from 30 to 2.5 µM using 24-well plates for adult worms and 96-well plates for Mfs. All assays were repeated at least three times, and the results obtained are the mean values at each concentration. IC_{50} determination was done graphically using GraphPad Prism software (version 6.0)

Isolation of Loa loa microfilariae for drug screens

Ethical clearance for the study was obtained from the Cameroon National Ethics Committee, while patients freely gave their written consent to participate in the study before recruitment. Clients living in the Edea Health District were invited to the Edea District Hospital for free screening. For the confirmation of the presence of *L. loa* Mfs, thick blood smear was prepared, stained with Giemsa and

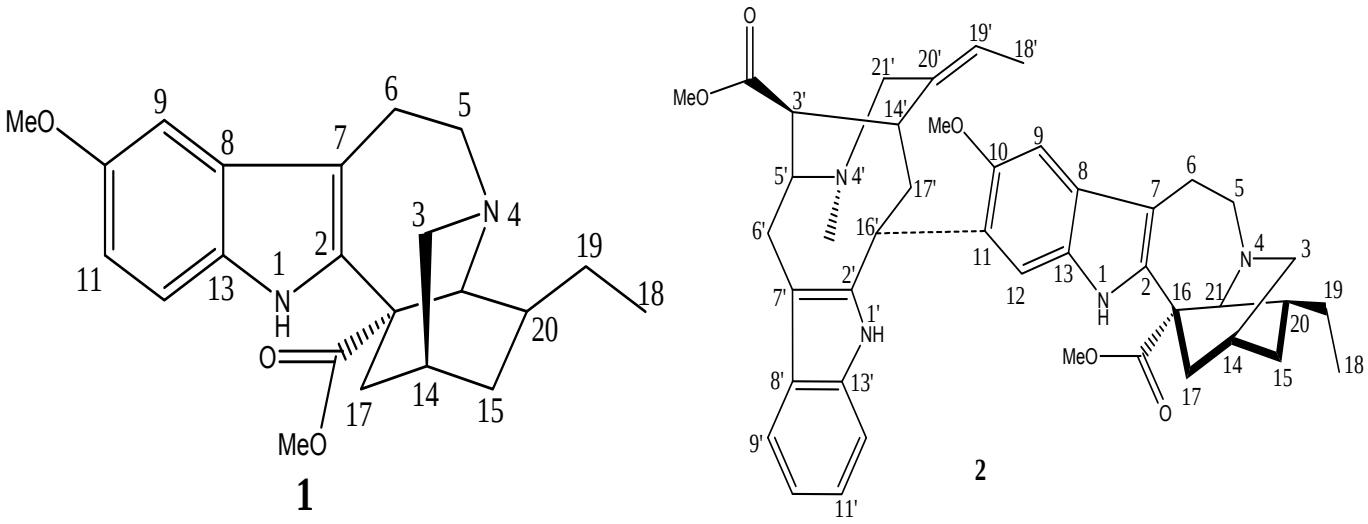

Figure 2. Structure of the compounds reported in this study. Compound 1 = voacangine and compound 2 = voacamine.

observed under light microscope. Patients having more than 1000 Mfs per ml of blood were recruited to donate blood. Ten millilitres of venous blood was collected in edetic acid (EDTA) tubes and gently mixed. Within 24 h, the sample was transferred to the laboratory at room temperature and allowed to stand for Mfs to settle. Slightly more than half of the plasma was recovered, and the remainder of the residual blood was mixed with two volumes of incomplete culture medium (liquid RPMI-1640 supplemented with 25 mM HEPES, 2 g/L sodium bicarbonate, 20 mM L-glutamate, 200 units/ml penicillin, 200 µg/ml streptomycin and 0.25 µg/ml amphotericin B, pH 7.4). The diluted sample was then carefully layered on ficoll in a 15 ml corning tube in appropriate ratio. Centrifugation at 805 × g (Eppendorf Centrifuge 5810R) for 15 min at room temperature resulted in layers with the top-most part containing the Mfs. Using a 10 ml serological pipette, the buffy coat and all the layers above the Red Blood Cell Count (RBC) pellets were transferred into another 15 ml tube and washed 3x in compliance and content monitoring (CCM) by centrifuging at 805 × g for 15 min. Recovered cells/Mfs were re-suspended in appropriate volume (10 ml) of incomplete medium supplemented with 5% New Born Calf Serum (NBCS). The Mfs could be kept temporary at room temperature for up to two hours without feeder layer cells or on in an incubator for up to ten days. Three portions of 10 µl of the Mfs preparation were pipette, and counted in order to check for the viability and determine the number of Mfs in each portion. Approximately, microfilariae were cultured at 10 to 30 Mfs per well in a 96-well standard culture plate containing LLC-MK2 cell layer as earlier done for *O. ochengi* mfs. All cultures were conducted at 37°C under an atmosphere of 5% CO_2 in humified air in a CO_2 incubator. Worm viability was assessed by mean motility scores on a scale of 0 (immotile), through 0.25 (only tail or head shaking occasionally), through 0.5 (whole body motile, but sluggishly or with difficulties), to 0.75 (almost vigorously motility) to 1 (fully vigorously motility). Scores were made every 24 h till 120 h using an inverted microscope. Any culture with microbial contamination was not considered.

Toxicity studies

Cytotoxicity was done as part of the Mfs assay on the active compound through observations on the monkey kidney epithelial cells on day 5. An examination of the deformities and degree of detachment of the monkey kidney cells was done. Dead or deformed cells were usually detached from the bottom of the vessel and were rounded in shape. The IC_{50} values for these mammalian cells were determined graphically using data from microscopy. The selectivity index (SI) values were calculated using the ratio: SI = IC_{50} of drug on mammalian cell/IC_{50} of drug on parasite (Mfs).

RESULTS

The stem bark of *V. africana* was extracted with methanol and subsequently liquid partitioned to obtain the methylene chloride extract. Vacuum liquid chromatography of this extract as described earlier, yielded two compounds (Figure 2) which were identified with the help of chemical tests, ^{1}H and ^{13}C NMR spectra and melting points as well as comparison of these data with published literature values. The compounds were tested *in vitro* on *O. ochengi* worms and the results are shown in Tables 1 to 4. At 30 µM both voacangine (compound 1) and voacamine (compound 2) were active against *O. ochengi* microfilariae (Mfs) and adult male worms, but moderately active against the female worms (Tables 1 and 2). They killed Mfs and adult male worms after 24 h incubation. After 120 h incubation, voacangine (compound 1) produced 50% inhibition of formazan formation in adult female worms while voacamine (compound 2) produced 65% inhibition. At 10 µM, FDA-approved auranofin, a gold conjugated compound, previously shown to be a macrofilaricide and a current arthritis drug (positive control) (Bulman et al., 2015) also produced 100% inhibition of formazan formation in adult female worms at 120 h of incubation. Table 3 summarises the inhibitory effect of voacangine and voacamine on *O. ochengi* microfilariae (Mfs), adult worms, and monkey kidney cells (MKCs), indicating the

Table 1. Effect of voacangine (compound 1) and voacamine (compound 2) on *O. ochengi* worms in primary screens.

*Test substance	% Microfilarial (Mf) motility reduction after 24 h	% Adult male worm motility reduction after 24 h	% Adult female worm death after 120 h	Comment(s)
Voacangine (30µM)	100	100	50	Active on Mfs and adult males; moderately active on adult females
Voacamine (30 µM)	100	100	65	Active on Mfs and adult males; moderately active on adult females
Ivermectin (10µg/ml)	100	NA	NA	Microfilaricidal
Auranofin (10 µM)	100	100	100	Macro- and microfilaricidal
2% DMSO	0	0	0	Inactive

*auranofin, was used as positive control for adult worm assay while ivermectin which is known not to kill adult worms was used for Mf assay. Dimethyl sulphoxide (DMSO) 2% (v/v) was used as negative control. Percentage adult female worm death corresponds to percentage inhibition of formazan formation. NA = Not applicable.

Table 2. Effect of voacangine (compound 1) and voacamine (compound 2) on *O. ochengi* worms in secondary screens.

Concentration (µM)	Mean percent inhibition of Mf motility after 24 h		Mean percent adult male wormmotility reduction after 24 h		*Mean percent adult femaleworm death after 120 h	
	voacangine	voacamine	voacangine	voacamine	voacangine	voacamine
30	100	100	100	100	50	65
20	100	100	100	100	42	50
15	100	100	75	100	0	40
10	75	100	50	100	0	34
5	50	75	25	75	0	0
2.5	0	50	0	25	0	0
10 Auranofin (positive control)	100		100		100	
2% DMSO (Negative control)	0		0		0	

*Percentage adult female worm death corresponds to percentage inhibition of formazan formation.

inhibitory concentration (IC) and selectivity index (SI) values. The compounds were moderately cytotoxic on the monkey kidney LLC-MK2 cells. To verify if any these related compounds could kill the microfilariae of the blood dwelling filaria, *L. loa*, which often pertubs mass drug administration for onchocerciasis with ivermectin due to the development of serious adverse events, the more effective compound, voacamine was tested on the parasite stage. After 48 h of drug testing *in vitro*, voacamine showed 100% inhibition of *L. loa* Mf motility at 5 µM drug concentration (Table 4).

DISCUSSION

Compound 1 was obtained as glass-like needles, mp 137 to 138°C. Its ^{1}H NMR spectrum revealed important signals at 7.87 ppm which was attributed to NH as well as others at 7.13, 6.91, 6.80, 3.85, 3.71, 3.54, 3.37, 3.22, 3.13 and 2.98 ppm which were attributed respectively to H-12, H-9, H-11, 10-C-OMe, -CO_2Me, H-21, H-6β, H-6α, H-5β and H-5α. Its ^{13}C NMR spectrum showed 22 signals notably at 175.5, 153.8, 137.3, 130.3, 129.0, 111.6, 110.9, 109.0 and 100.5 which were assigned respectively to C=O of the ester, C-10, C-2, C-13, C-8, C-11, C-12, C-7, and C-9. These values and all other signals were in close agreement with those reported for voacangine (Pereira et al., 2008; Zocoler et al., 2005). Compound 2 was obtained as a white powder, with melting point of 223°C. Its ^{1}H NMR spectrum showed signals at 7.73, 7.56, 7.49, 7.06, 7.05, 7.04, 6.93, 6.74, 5.32, 5.15 and 4.05 which were attributed respectively to HN-1', H-9', HN-1, H-10', H-11', H-12', H-9, H-12, H-19', H-16', and H-5' amongst others characteristic of voacamine (Medeiros

Table 3. IC_{50}, IC_{100} and selectivity indices (SI) of voacangine and voacamine on *O. ochengi* microfilariae and adult male worms

Compound	IC_{50} or CC_{50} (µM)				IC_{100} (µM)				SI	
	Mf	AM	AF	MKC	Mf	AM	AF	MKC	Mf	AM
Voacangine	5.49	9.07	-	≥30	10	20	>30	-	5.46	3.3
Voacamine	2.49	3.45	-	≥30	10	10	>30	-	12.04	8.69

Mf = microfilariae; AM = Adult male worms; AF = Adult female worms; MKC = Monkey kidney cells (LLC-MK2); Selectivity Index (SI) = CC_{50} MKC/IC_{50} worm.

Table 4. Effect of voacamine on *L. loa* microfilariae worm motility.

Incubation time (H)	Percent inhibition of *L. loa* microfilarial motility at different concentrations (in µM) of voacamine					
	30 µM	20 µM	15 µM	10 µM	5 µM	2.5 µM
6	0	0	0	0	0	0
12	0	0	0	0	0	0
18	0	0	0	0	0	0
24	0	0	0	0	0	0
30	75	75	50	50	0	0
36	100	100	100	100	50	0
42	100	100	100	100	75	0
48	100	100	100	100	100	0
72	100	100	100	100	100	0
96	100	100	100	100	100	0
120	100	100	100	100	100	0

et al., 1999). Its ^{13}C NMR spectrum portrayed signals for 42 carbon atoms at 175.3, 171.6, 150.9, 137.9, 137.1, 135.8, 130.3, 129.9, 129.8, 127.3, 121.5, 118.8, 118.5 and 117.4 which were attributed, respectively to C-22, C-22', C-10, C-2', C-2, C-13', C-13, C-8', C-11, C-8, C-11', C-10', C-19' and C-9' characteristic of the dimer voacamine (Medeiros et al., 1999). They were also identified by TLC comparison with authentic samples of the compounds in the laboratory. It has been suggested that medicinal plants used in folk medicine to treat parasitic diseases may provide an alternative source of *Onchocerca* macrofilaricides (Comley, 1990). Thus, our quest for lead compounds from medicinal plants for the development of such a drug led to the isolation of voacangine and voacamine from the stem bark of *V. africana*.

This study reports for the first time, the efficacy of these natural products against microfilariae and adult *O. ochengi* worms. The control drug, auranofin is a gold conjugated compound that has been used in the treatment of rheumatoid arthritis in humans. Previous studies on drug repurposing (Bulman et al., 2015) have shown that auranofin is effective against *O. ochengi* worms *in vitro*. *O. ochengi* is reported to be the closest relative of *O. volvulus* and best model for anti-*O. volvulus* drug screens; both share the same vector and form similar sub-cutaneous nodules (Achukwi et al., 2000; Trees et al., 2000). Thus, it is likely that a drug that kills the *O. ochengi* worm will also be effective against *O. volvulus*. This was demonstrated, for example, with ivermectin, moxidectin and tetracyclines. Voacamine was more active in the *in vitro* assays than voacangine on Mfs and adult male worms. At this time, their targets in the *O. volvulus* parasite is not known. Their presence in the plant may explain in part the apparent anti-*Onchocerca* activity of its decoctions as used in the traditional treatment of onchocerciasis in the NW Region of Cameroon. Although, the compounds turned out to be inefficient at killing the adult female *Onchocerca* worms in the assays, it is hoped that if developed as drugs, they could break transmission of the disease since in the absence of male worms, adult females will not produce the microfilariae known to be responsible for most of the debilitating effects of onchocerciasis. However, people using this plant as herbal medicine to treat onchocerciasis should be screened for *L. loa* co-infection or observed closely since voacamine has been demonstrated in this study to kill *L. loa* microfilariae. This is to avoid the adverse reactions observed with some highly co-infected patients on ivermectin treatment in which *L. loa* microfilariae are killed, leading to serious adverse effects, including encephalopathy and death (Bourguinat et al., 2010).

The effect of voacamine on *L. loa* comes to add to

some of the drawbacks of the arbitrary use of medicinal plants in the treatment of diseases. Although, in reality, such adverse events are generally undocumented. Voacamine has also been isolated from other plants such as *P. fuchsiaefolia* and approved for the treatment of malaria in several African countries (Stella and Soriba, 2007). Voacangine on the other hand has been reported for anti-plasmodial activity (Federici et al., 2000) and anti-angiogenesis activity *in vitro* and *in vivo* (Yonghyo et al., 2011).

Conclusion

Our quest for leads from medicinal plants for the discovery of drugs against onchocerciasis led to the isolation and characterization of two compounds: voacamine and voacangine from the stem bark of *V. africana*. These compounds are reported for the first time for anti-*Onchocerca* activity *in vitro*. The results support the traditional use of *V. africana* in the treatment of human onchocerciasis, but its usage should preferably be limited to regions with low levels of *L. loa* co-endemicity.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Achukwi MD, Harnett W, Renz A (2000). *Onchocerca ochengi* transmission dynamics and the correlation of *O. ochengi* microfilaria density in cattle with the transmission potential. Vet. Res. 31(6):611-621.

Ameyaw Y, Duker-Eshun G (2009). The alkaloid contents of the ethno-plant organs of three antimalarial medicinal plant species in the Eastern Region of Ghana. Int. J. Chem. Sci. 7(1):48-58.

Bourguinat C, Kamgno J, Boussinesq M, Mackenzie CD, Prichard RK, Geary TG (2010). Analysis of the mdr-1 gene in patients co-infected with *Onchocerca volvulus* and *Loa loa* who experienced a post ivermectin serious adverse event. Am. J. Trop. Med. Hyg. 83(1):28-32.

Bulman CA, Bidlow CM, Lustigman S, Cho-Ngwa F, Williams D, Rascón AA, Tricoche N, Samje M, Bell A, Suzuki B, Lim KC, Nonglak S, Prasit S, Wolfe AR, Knudsen GM, Chen S, Wilson C, Kean-Hooi A, Arkin M, Gut J, Franklin C, Marcellino C, McKerrow JH, Debnath A, Sakanari JA (2015). Repurposing Auranofin as a Lead Candidate for Treatment of Lymphatic Filariasis and Onchocerciasis. PLoS Negl. Trop. Dis. 9(2):e0003534.

Cho-Ngwa F, Daggfeldt A, Titanji VPK, Gronvik K (2005). Preparation and characterization of specific monoclonal antibodies for the detection of adult worm infections in onchocerciasis. Hybridoma 24(6):283-290.

Comley JC, Rees MJ, Turner CH, Jenkins DC (1989). Colorimetric quantitation of filarial viability. Int. J. Parasitol. 19(1):77-83.

Comley JCW (1990). New macrofilaricidal leads from plants. Trop. J. Parasitol. 59(1):77-83.

Federici E, Palazzino G, Nicoletti M, Galeffi C (2000). Antiplasmodial activity of the alkaloids of *Peschiera fuchsiaefolia.* Planta Med. 66(1):93-95.

Gardon J, Gardon-Wendel M, Demanga N, Kamgno J, Chippaux J, Boussinesq M (1997). Serious reactions after mass treatment of onchocerciasis with ivermectin in an area endemic for *Loa loa* infection. Lancet 350(9070):18-22.

Lizotte-Waniewski M, Tawe W, Guiliano D, Lu W, Williams SA, Lustigman S (2000). Identification of potential vaccine and drug target candidates by expressed sequence tag analysis and immune screening of *O. volvulus* larval cDNA libraries. Infect. Immun. 68(6):3491-3501.

Ndjonka D, Agyare C, Lüersen K, Djafsia B, Achukwi D, Nukenine EN, Hensel A, Liebau E (2011). *In vitro* activity of Cameroonian and Ghanaian medicinal plants on parasitic (*Onchocerca ochengi*) and free-living (*Caenorhabditi selegans*) nematodes. J. Helminthol. 85(3):304-312.

Medeiros LBW, Vieira JCI, Mathias L, Braz-Filho R, Leal ZK, Rodrigues-Filho E, Schripsema J (1999). Two known bis-indole alkaloids isolated from *Tabernaemontana laeta*: complete ^{1}H and ^{13}C chemical shift assignments. Magn. Reson. Chem. 37:676-681.

Pereira SP, Suzelei CF, Oliveira PVA, Brevese CMS, Pereira SIV (2008). Chemical constituents from *Tabernaemontana catharinensis* root bark: A brief NMR review of indole alkaloids and *in vitro* cytotoxicity. Quim. Nova 31(1):20-24.

Stella L, Soriba C (2007). Use of voacamine and related compounds in the treatment of malaria. US Patent 2007/0032460 A1.

Trees AJ, Graham SP, Renz A, Bianco AE, Tanya V (2000). *Onchocerca ochengi* infections in cattle as a model for human onchocerciasis: Recent developments. Parasitolology 120:S133-S142.

Wogu MD, Okaka CE (2008). Prevalence and socio-economic effects of onchocerciasis in Okpuje, Owan West Local Government Area, Edo State, Nigeria. Int. J. Biomed. Health Sci. 4(3):113-119.

World Health Organization (WHO) (2007). Global Initiative for the Elimination of avoidable Blindness: Action plan 2006 – 2011 p 29.

Yonghyo K, Hye JJ, Kwon HJ (2011). A natural small molecule voacangine inhibits angiogenesis both *in vitro* and *in vivo*. Biochem. Biophys. Res. Commun. 417(2012):330-334.

Zocoler AM, Oliveira JBA, Sarragiotto HM, Grzesiuk LV, Vidotti JG (2005). Qualitative determination of indole alkaloids of *Tabernaemontana fuchsiaefolia* (Apocynaceae). J. Braz. Chem. Soc. 16(6B):1372-1377.