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Anthelmintic Activity, Acute Toxicity of *Anacardium occidentale L.* (Anacardiaceae) on *Onchocerca ochengi* and *Caenorhabditis elegans*

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Authors' contributions

This work was carried out in collaboration between all authors. Author HNTM collected the plants, prepared the extracts performed the experiments, analyzed the results and drafted the manuscript. Author DN did the conception, sourced for funding, designed and directed the experiments and corrected the manuscript. Author RM supervised the experiments and corrected also the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: The objective was to evaluate the anthelmintic activity of the ethanolic stem bark of *A. occidentale.*
Study Design: Ethanolic extract of stem barks of *Anacardium occidentale* was tested *in vitro* against the cattle filarial parasite *Onchocerca ochengi* and the wild type, the drug resistant strains of the free living nematode *Caenorhabditis elegans*.
Place and Duration of Study: The work took place at the Laboratory of Applied Zoology of the Faculty of the University of Ngaoundere between May 2016 and March 2017.
Methodology: Adults of *O. ochengi* were incubated in RPMI-1640 medium supplemented with penicillin/streptomycin and plant extract or drugs. *C. elegans* was incubated at 20°C with plant

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extract or drugs in M9-medium. Worm mortality was determined biochemically by MTT/formazan colorimetry after 24, 48 and 72 h. Ivermectin and levamisole were used as positive control and DMSO as negative control. Acute toxicity on mice (*Mus musculus*) and phytochemical compounds were also determined.

Results: Ethonolic stem bark extract (SBE) of *Anacardium occidentale* was active against worms. *O. ochengi* was the most affected by the extract with the LC_{50} values of 88 ± 0.16 µg/mL, 3.18 ±0.17 µg/mL and 2.76 ± 0.10 µg/mL respectively after 24, 48 and 72h of incubation. The quantitative phyto-constituents screening has revealed the presence of tannins, phenolic acids, saponins and flavonoids. Tannins (107.16 mg GAE/ 100 g) were the most quantified compound followed by phenolic acid (26.23 mg/g GAE), saponins (9.33 mg/g) and flavonoids (0.16 mg of rutin/100 g). The acute toxicity study has demonstrated that at short term (14 days), oral intake of ethanolic stem bark extract was not toxic up to a dose of 1600 mg/kg by weight.

Conclusion: This study justifies the use of this plant by traditional health practitioners in managing the disease, and also suggests a source of compounds against *Onchocerca volvulus*.

Keywords: Onchocerca ochengi; Caenorhabditis elegans; drug resistant strains; levamisole; ivermectin.

ABBREVIATIONS

DMSO: Dimethyl Sulfoxide; O: Onchocerca; C: Caenorhabditis; SBE: Stem Bark Extract; PBS: Phosphate Buffered Saline; SE: Standard Error of Mean; SPSS: Statistical Package for the Social Sciences; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; NGM: Nematode Growth Medium; OCP: Onchocerciasis Control Program; OECD: Organization for Economic Cooperation and Development; HNC: National Herbarium of Cameroon.

1. INTRODUCTION

Onchocerciasis or 'river blindness' is a parasitic disease caused by the filarial worm *Onchocerca volvulus* and transmitted by blackflies of the genus *Simulium damnosum* [1]. The female worm can produce millions of embryos each day during her lifetime (10-15 years) [2]. The microfilariae live for around 1 year, reside primarily in the sub-epidermal layer of the dermis and can invade the eyes [3]. It is the world's second leading cause of blindness, after trachoma being number one. Estimations show that 37 million people are infected presently with onchocerciasis, more than 250,000 are blind and 500,000 have some degree of visual impairment [2,4]. Onchocerciasis control started in 1974 with the Onchocerciasis Control Programme in West Africa (OCP) which conducted large scale larviciding of vector breeding sites in the West African Savannah. The vector-control based strategy was complemented with mass drug administration and after ivermectin (Mectizan®) had been registered and Merck decided to donate ivermectin for onchocerciasis control for as long and in the amounts needed [5]. In spite of the successes reported in reducing the disease burden, total elimination of onchocerciasis has not been achieved and remains always a major public health problem [6]. When ivermectin, a macrocytic lactone, is present in sufficiently high concentrations to cross the blood–brain barrier, it can cause neurologic signs [7]. Suboptimal response to ivermectin has been registered in others studies [8]. In addition, it has been registered that the efficacy of ivermectin is essentially limited to microfilariae and onchocerciasis control based on a single drug, revealed a resistance of *O. volvulus* [9]. Thus, there is an urgent need to search a new, safe and efficacious macrofilaricidal and microfilaricidal drugs. Medicinal plants have been considered as an alternative source of compounds that are biodegradable into non-toxic products and sustainable methods readily adaptable to rural farming communities [10]. In most developing countries, small holder farmers have limited access to such drugs and veterinary services due to either high cost or unavailability [11]. In an attempt to contribute to search an anti-onchocercal drug, *Anacardium occidentale* was selected based on information obtained from traditional healers, for the treatment of human and livestock parasites in the northern part of Cameroon. *A. occidentale* L. commonly known as cashew tree is native from Brazil. *A. occidentale* belongs to the family of the flowering plants- Anacardiaceae [12]. Its English name-Cashew is the Portuguese name for the fruit of

the cashew tree Caju, which itself is derived from the indigenous Tupi name, *acaju* and means 'nuts that produces itself' [13]. Its local name is kaâkoy. The *Anacardium* actually refers to the shape of the fruit which looks like an inverted heart (*ana* means 'upwards' and *cardium* means 'heart') [12]. The family contains 73 genera and about 600 species [14]. It is a multipurpose tree of the amazon that grows up to 15 m high and is characterized by a leafy tree [12]. *A. occidentale* is used to treat malaria [15], leishmaniasis and trypanosomiasis [16,17]. As reported by Kunle et al. [18] cashew had been used extensively in the treatment of malaria. Thus, the anthelmintic activity of the ethanolic stem bark of *A. occidentale* were evaluated on the parasite of cattle, *Onchocerca ochengi* and on different strains of the free living nematode *Caenorhabditis elegans*. Thus, the anthelmintic activity of the ethanolic stem bark of *A. occidentale* were evaluated on the parasite of cattle, *Onchocerca ochengi* and on different strains of the free living nematode *Caenorhabditis elegans*.

2. MATERIALS AND METHODS

2.1 Sample Collection and Identification of *Anacardium occidentale*

Plant materials were collected in June 2013 at Garoua (Nord-region of Cameroon: latitude 09°23'9.46'' N, longitude 13°20'18'' E and altitude 199 m above sea level). Botanical's plant identification was done at the national herbarium of Cameroon in Yaounde, where voucher specimens were kept and registered under number 02 (65604/HNC).

2.2 Processing of Plant Extract for Tests

The extract was performed using the modified method described by Ojezele et al. [19]. Briefly, stem barks of *A. occidentale* were harvested early in the morning, washed and dried in the shade at room temperature. The dry part was sieved on a 0.5 mm mesh screen. Hundred (100) mg of the powder was weighed and macerated for 48 hours in 100 mL of ethanol (70%). The mixture was filtered over filter papers No. 413 (VWR International, Darmstadt, Germany). The filtrate was then concentrated under reduced pressure by rotary evaporation (BUCHI Rotavapor R-200, Switzerland) at 40°C. Residual solvent was removed by drying in a sweating-room at 35°C and the extract was weighed and stored at + 4°C. For further investigation, the dried stem barks of *A. occidentale* was dissolved in dimethyl sulfoxide (DMSO) and RPMI (Roosevelt Memorial Park) to a final concentration of 100 mg/mL, filtered and aliquoted. Its anthelmintic activity was evaluated on *C. elegans* strains and *O. ochengi*.

2.3 Preparation of Positive Control

Ivermectin and levamisole used as positive controls were dissolved in 10% dimethyl sulfoxide (DMSO) and diluted with RPMI. The final concentrations of both drugs for RPMI-stock solutions were 2 mg/mL. DMSO was used as negative control. The maximal concentration of DMSO used was lower 1%.

2.4 Extraction, Isolation and *In vitro* Assay of *Onchocerca ochengi*

The isolation of *O. ochengi* adult worms was done according to the modified process used by Cho-Ngwa et al. [20]. Portions umbilical cattle skin with palpable nodules bought from local slaughterhouses were washed, drained and sterilized with 70% ethanol. Nodules were isolated from the skin, using a scalpel, by making a slight tear on the inner face of the skin. The isolated nodules were placed in a solution of PBS (Phosphate Buffered Saline) for manual dissection. The viability of worms retained for the assay was ascertained using inverted microscope (Euromex, Holland) examination and damaged worms were discarded.

After isolation, damaged worms and worms from putrefied nodules were discarded. The test was processed following methods established by Ndjonka et al. [21]. The viability of worms retained for the assay was ascertained by microscopic examination of adult worm motility using a dissecting microscope (Euromex, Holland). The normal worms were carried under the hood and washed 3 times in the plates containing sterile PBS and then twice in the RPMI (Roosevelt Memorial Park Medium-1640, SIGMA, USA) which is the culture medium. Following the modified protocol of Borsboom et al. [22], worms were incubated in different concentrations of the plant extract or drug prepared solutions in RPMI 1640 supplemented with penicillin/streptomycin (100 U/100µg/mL). The assay was conducted in 96-well microtitre plates at 37°C under an atmosphere of 5% CO_2

in air for 3 days. The mortality was determined after 24 h, 48 h and 72 h.

2.5 Culture, Synchronization and Assays of *Caenorhabditis elegans*

The different strains of *C. elegans* (*C. elegans* wild type (N2 Bristol) and the mutant strains CB211 lev-1(e211) IV, VC722 glc-2(ok1047) I) used in this work were obtained from the Caenorhabditis Genetic Centre (CGC, Minneapolis, MN, USA). Cultures were realized as describe by Ndjonka et al. [21]. Worms were grown at 20^{o} C under standard monoxenic conditions on NGM-agar (Nematode Growth Medium: 2.5 g peptone from casein, 3 g NaCl, 17 g agar, 0.5% cholesterin, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM KH_2PO_4/K_2HPO_4 in 1 l of water) in Petri dishes seeded with *Escherichia coli* OP 50 to serve as food source. Petri dishes of 85 mm with gravid adult worms were selected for age-synchronization [23].

C. elegans worms age-synchronized were obtained from eggs and isolated by bleaching [24]. The content of the plate was rinsed for 5 to 6 minutes. The supernatant was recovered in eppendorf tubes then centrifuged at 5500 rpm for 1 min at 4°C. The supernatant was treated for 6 min with a mixture of distilled water, alkaline sodium hypochlorite (4% NaOCl) and sodium hydroxide (10 M NaOH). Then, the mixture was centrifuged at 6500 rpm for 1 min at 4°C. The supernatant was discarded and the pellet with viable eggs was rinsed 3 times in 1mL M9 buffer. During each rinse, the mixture was centrifuged at 5500 rpm for 1 min 4°C. The pellet was kept in 200 µL M9 buffer during the last rinse and then introduced in axenic liquid medium (3% (w/v) yeast extract, 3% (w/v) soy peptone, 1% (w/v) glucose, 0.5 mg/mL cholesterol and 0.5 mg/mL bovine hemoglobin) supplemented with 100 U/mL penicillin and 100 g/mL streptomycin. After 48 hours of incubation at 20°C, the young adults or L4 larvae of the same age were used in 24-well plates to test plant's toxicity [23,25].

The synchronized worms (ten young L4 adults per well) were transferred and incubated in M9 buffer with different concentrations of plant extracts or drug in the 24-well plates. Each well received 500 µL of M9 buffer to which the volume of extract's stock solution corresponding to each concentration was added. Negative control plates did not contain plant extracts or drug. Positive controls were performed with levamisole or ivermectin. Three trials were conducted for each concentration and the worms were incubated at 20°C. The mortality was determined after 24 h and 48 h and 72 h [26].

2.6 Biochemical Determination of Worm Viability

To recognize the dead or living worms, the worms were removed from their wells, then washed with PBS (phosphate-buffered saline) and subjected to the MTT colorimetric test. The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) is a pale yellow compound which is reduced to a dark blue product, formazan by the living cells of the worms. Single intact worms are placed in each well of a 48-well plate (Falcon, UK) containing 500 µL of a solution consisting of 5 µL MTT (0.5 mg/mL) and 5 µL of RPMI, then incubated at 37°C and observed after 30 min under a binocular microscope. Thus after incubation, when the worms were alive they were colored blue because the MTT was reduced to formazan. Dead worms do not reduce MTT to formazan but simply take on the yellow color of MTT. All the MTT assays were done in the dark since the MTT reagent is sensitive to light [27].

2.7 Quantitative Determination of Secondary Metabolites

The tannins determination was carried out following the modified method of Pradeepa et al. [28]. Briefly 200 µL of a solution of the sample were mixed with 35% (w/v) of Na_2CO_3 and 100 µL of Folin-Ciocalteu (FC) reagent was added. The whole was mixed by vortexing for one minute and incubated five minute at room temperature (25 to 30°C). The absorbance was then read at 640 nm.

The process described by Madhu et al. [29] was used to evaluate flavonoids content. Briefly, to 0.1 g of the extract, 2 mL of the extract solvent made of 140:50:10 methanol-distilled water-acetic acid were added. The obtained solution was filtered using a Whatman paper and an equal volume of extraction solvent was added. 250 µl of the filtrate was transfered to a 5 mL tube and completed to 5 mL with distilled water which constitutes the analysis solution (A solution). For titration, to 5 mL of the A solution, 200 µl of distilled water and 500 µl of aluminium Chloride solution (133 mg of $AlCl_3$ and 400 mg of sodium acetate in 100 mL distilled water) were added and the solution mixed by vortexing. A

rutin titration curve was set at the absorbance of 430 nm and the flavonoids amount was expressed as mg of rutin/100g of dry material.

The total phenolic content of extract was determined by Folin-Ciocalteu reagent (FCR) technical employed by Junaid et al. [30] with minor modifications. In the procedure, the gallic acid content was determined using different concentrations of its aqueous solution. A titration curve of gallic acid at a wave length of 765 nm was then drawn from the evaluated absorbance. Briefly, to 50 µl of the sample-solution, 200 µL of Na_2CO_3 and 250 µL of a 1/10 (v/v) of FC reagent were successively added. The obtained solution was agitated manually for one minute and incubated in dark at 40°C for 30 minutes. The absorbance was determined at 765 nm using a spectrophotometer (UV-biowave Cambridge, England). The phenolic acids content was calculated from the standard curve of gallic acid titration's equation (linear regression equation). Tannins and phenolic acids contents were expressed as equivalent of gallic acid per gram of dry plant material (mg GAE/100 g).

The saponins content was determined following the modified method described by Junaid et al. [31]. To 0.1 g of the extract, 1 mL of distilled water was added and vigorously shaken for 30 min. The height of moss was measured by a ruler and quantified like following: Saponin (mg) = [(0.432) (height of moss in cm after 5 to 10s) + 0.008] / (weight of sample in gram).

2.8 Oral Acute Toxicity Study: Experimental Design

Mice weighing between 25 and 30 g were purchased from LANAVET (National Veterinary Laboratory) in the North-region (Cameroon). Animals were housed and maintained under ambient temperatures (25°C ± 3°C) where they were acclimatized to standard laboratory conditions for 7 days. The acute oral toxicity study was conducted in compliance with OECD guideline 423 which stipulate the use of only three animals per dose (OECD 423, Paragraph 23) [32]. Three groups of three animals were fasted overnight (12 h) and weighed. Test doses of stem bark ethanolic extract of *A. occidentale* were calculated in relation to the body weight of every fasted animal and administered via oral gavage.

Briefly, in the first phase, mice received 1000 mg/kg of extract and were observed for physical signs of intoxication every 4 h for a period of 24 h. The control group received 1% DMSO diluted in distilled water. The next dose was scheduled based on the number of death recorded in stage one and animals (n=1) received 1300, 1600 and 1900 mg/kg of extracts. Then, the animals were regularly and individually observed for behavioral changes and general toxicity signs after dosing for the first 24 h, with special attention being given during the first 4 h and thereafter twice daily for a continuous period of 14 days. The numbers of deaths within this period were recorded [33].

2.9 Statistical Analysis

The Graphprism program 5.0 software was used to set out the curves. The LC_{50} values were determined by Probit Analysis method using SPSS 16.0.Turkey's test at P= 0.05 was applied for mean separation. The LC_{50} values were expressed as mean ± standard deviation (mean of 3 replicates).

3. RESULTS

The stem bark extract (SBE) of *A. occidentale*, levamisole and ivermectin were nematocidal in a time and concentration-dependent manner as shown in Figs. 1 and 2. There was not mortality recorded in 0.5% of DMSO used as negative control.

3.1 Activity of SBE and Ivermectin on *O. ochengi*

The Fig. 1 shows mortality rate of *O. ochengi* and *C. elegans* wild type as a function of ethanolic SBE of *A. occidentale*. The mortality rate increases quickly at 5 µg/mL and 25 µg/mL respectively in Fig. 1A and 1B. At the three time points, the SBE of *A. occidentale* was lethal to the incubated worms. The mortality reached 100% after 72 h incubation at concentrations 12.5 µg/mL and 100 µg/mL respectively for *O. ochengi* and wild type *C. elegans* as displayed in Fig. 1. The LC_{50} values at three different time points are summarize in Table 1.

O. ochengi was most affected by the SBE with the LC_{50}.values between 2.76 and 5.88 µg/mL. However, compared to levamisole and ivermectin, SBE was less efficient. The efficacy of SBE on *C. elegans* affected more the wild type than resistant strains with LC_{50} varying between 3.17 to 15.22 µg/mL. Additionally the crude extract displayed a remarkable activity against all

the two worms with LC_{50} values between 2.76 and 15.22 µg/mL. The crude extract hence exhibits nematocidal activity at LC_{50} below 16 µg/mL for the three exposure period (24 h, 48 h and 72 h). Table 1 shows that there is no significant difference (p=0.001) in the effect of the stem bark on the two worms after 72 h incubation. Compared to ivermectin and levamisole at any time, the crude extract exhibited a slightly higher LC_{50} on *C. elegans* and *O. ochengi*. The crude extract therefore causes a much lower mortality than the positive control. However, the difference was not significant between LC_{50} values at 48h. In each row, the results showed that efficacy of ivermectin and SBE was the same at 48h and 72h of incubation of worms.

The Fig. 2 shows the mortality of levamisole- (Fig. 2A) and ivermectin- strain (Fig. 2B) of *C. elegans* as a function of increasing concentrations of ethanolic SBE of *A. occidentale* at three different time points of incubation. Both figures reveal that SBE was lethal to worms. At all three time points, immediately after incubation, the mortality of worm increases quickly then reaches 100% at 72h. Total mortality was not observed at 24h with levamisole and ivermectin-resistant strains. The LC_{50} values are summarized in Table 1 which shows that the crude extract is most potent on the wild type strain compared to the levamisole-and the ivermectin-resistant strains. However the stem bark crude extract exhibited the mortality effect on levamisol- and ivermectin-resistant strains of *C. elegans* with 100% mortality at higher concentrations (>200 µg/mL) after 72 h (Fig. 2). Additionally ivermectin-resistant strain VC722 (LC_{50} 47.05, 26.62 and 13.21 µg/mL respectively at 24, 48 and 72 h) and levamisol-resistant strain CB211 (LC_{50} 25.74, 10.93 and 5.77 µg/mL respectively at 24, 48 and 72 h) are sensitive to the crude stem bark compared to the two reference drugs (ivermectin and levamisol). The difference was not significant at three different time points between LC_{50} values of ethanolic SBE on levamisole (CB211) and ivermectin-resistant strains (VC722).

3.2 Phytochemical Compounds from SBE of *A. occidentale*

Table 2 presents the results of the phytochemical content in the SBE of *A. occidentale*. Tannins, flavonoids, saponins and phenolic acids were quantitatively determined. Tannins and phenolic acids were the most abundant compound content with 107.16 ± 0.56 mg/kg and 26.33 ± 0.04 mg/kg respectively. Flavonoids were the less quantified content with 0.16 ± 0.04 mg/kg.

3.3 Acute Toxicity Assays on Mice

The result of acute toxicity of the SBE of *A. occidentale* has presented one death at 1900 mg/kg after 24 h. However, some toxic signs (locomotion, convulsions, loss of appetite and sneezing) were registered. No toxic signs and death were manifested in control during all the period of study.

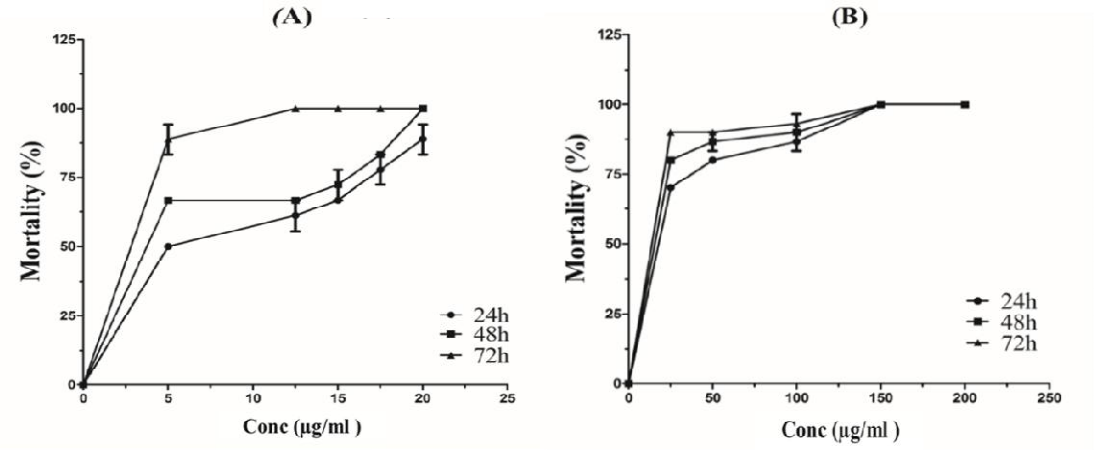

Fig. 1. Activity of SBE of *A. occidentale* on *Onchocerca. Ochengi* (A) and on wild type of *C. elegans* (B). SBE affected the survival of the two worms in a time and concentration-dependent manner

Data are mean ± SDM from three independent triplicate experiments

Table 1. LC_{50} values of the ethanolic SBE of *Anacardium occidentale* and positive controls tested against *O. ochengi* and *C. elegans* (wild type and drug resistant strains) after 24, 48 and 72 h exposure

Worms	LC_{50} (µg/mL) (95% FL) Khi-carré								
	24 h			48 h			72 h		
	Stem bark	Ivermectin	Levamisole	Stem bark	Ivermectin	Levamisole	Stem bark	Ivermectin	Levamisole
O. ochengi	5.88± 0.16^{aA} (2.97-7.95) 64.04***	0.18±0.06^{cA} (0.15-1.44) 137.25***	nd	3.18±0.17^{aAC}**ns** (0.03-6.09) 109.41***	0.10±0.07^{aB} (0.05-0.13) 45.97***	nd	2.76±0.10^{aC} (1.00-3.57) 19,14^{ns}	0.07±0.04^{bB} (0.04-0.84) 47.89***	nd
C. elegans WT	15.22 ± 0.15^{aA} (7.12-22.60) 54.68***	2.75 ± 1.42^{bA} (1.54-3.56) 15.25^{ns}	5.82 ± 0.31^{aA} (4.97-6.51) 17.97^{ns}	8.63 ± 0.16^{acA} (2,51-15.19) 41.54***	2.45 ± 0.41^{cA} (0.95-3.59) 20.25*	3.94 ± 0.64^{cB} (2.76-4.73) 19.21^{ns}	3.17 ± 0.18^{aA}ns (0.03-9.96) 50.50***	2.23 ± 1.35^{aA} (1.22-3.88) 43.54***	3.56 ± 1.16^{aB} (2.74-4.74) 38.80***
C. elegans CB211	25.74 ± 0.08^{aA}**ns** (16.48-35.34) 38.16***	nd	>100	10.93 ± 0.08^{aA}**ns** (4.54-18.04) 64.40***	nd	>100	5.77 ± 0.09^{aA}**ns** (1.03-12.04) 103.89***	nd	>100
C. elegans VC722	47.05 ± 0.11^{aA} (34.02-60.22) 49.08***	>100	nd	26.62 ±0.11^{aA}**ns** (14.59-37.41) 52.79***	>100	nd	13.21 ± 0.13^{aA}**ns** (4.14-22.33) 56.46***	>100	nd

The LC_{50} values are expressed as mean ± standard deviation (SD) of mean of 3 replicates. Small letters compare means in a column and capital letters means in a row. Different letters indicate significant difference at p = 0.05. nd: not determinated; FL: Fiducial Limit; ns: not significant;
**p = 0. 05; ***p = 0.001*

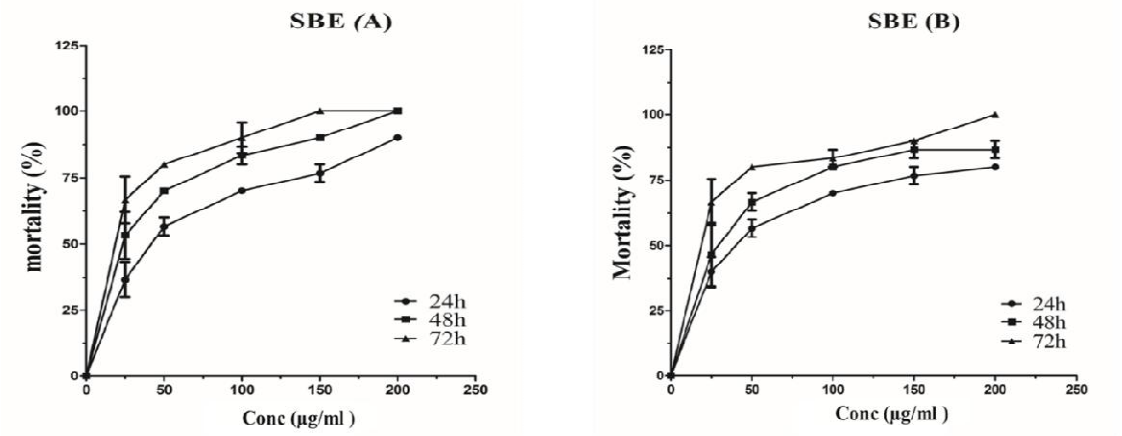

Fig. 2. Activity of SBE of *A. occidentale* on levamisole (A) and on ivermectin (B) resistant strains of *C. elegans*. SBE affected the survival of the two mutant strains of *C. elegans* in a time and concentration-dependent manner

Data are mean ± SDM from three independent triplicate experiments

Table 2. Quantity (mg) of phytochemical compounds (tannins, flavonoids, saponins and phenolics)

A. occidentale	Tannins (mg/g GAE)	Flavonoids (mg rutin/100 g)	Saponins (mg/g GAE)	Phenolic acids (mg/g GAE)
Stem barks (100 g)	107.16 ± 0.56	0.16 ± 0.04	9.33 ± 0.06	26.33 ± 0.04

For each 100 grams of the ethanolic SBE of Anacardium occidentale. GAE: Gallic Acid Equivalent. Figures are expressed as mean ± SD

4. DISCUSSION

The objective of the present study was to determine the *in vitro* anthelmintic activity of the ethanolic SBE of *A. occidentale* against the nematode parasite of cattle *O. ochengi* and *C. elegans* (wild type and mutants). Levamisole and ivermectin were used as positive control. The assays were performed in triplicate, and the results (Fig. 1 and Fig. 2) indicated that the efficacy of the SBE is different for the two worms. The ethanolic SBE of *A. occidentale* and drugs showed interesting results, they were lethal to both nematodes. The mortality of worms was concentration and time-dependents. *O. ochengi* and *C. elegans* are affected by the SBE with lower LC_{50}.values. Previously, several others studies have reported the activity of *A. occidentale* [34-35]. It has been used in the treatment of intestinal parasites [34]; and also used to treat gastrointestinal disorders [36]. In addition, a compound named anacardic acid (6-pentadecylsalicylic acid) isolated from *A. occidentale* was also demonstrated by Tan and Chan [35] to be lethal against *Staphylococcus* species.

The most targeted parasite species with the use of *A. occidentale* are helminths namely dracunculiasis, schistosomes and trypanosomes [37]. Parallel studies carried out with different plants [6,20,21,23,38,39,40] have demonstrated anti-Onchocerca and anthelmintic activities.

Recently in a study**,** Rajesh et al. [34] has mentioned that an activity of a plant extract depends on the availability of bioactive compounds, secondary metabolites like tannins, saponins, terpenoids, alkaloids and flavonoids. The SBE of *A. occidentale* contains almost the same secondary metabolites namely tannins, saponins, flavonoids and phenolic acids which might be toxic for the worms and responsible for the anthelminthic activity observed. According to Hoste et al. [41], tannins are able to bind to glycoproteins on the cuticle of parasites. The similar mode of action of tannins on nematodes was also reported with synthetic phenolic anthelmintics (oxyclozanide, niclosamide, nitroxynil) [42], as these drugs interfere with energy generation in helminth parasites by uncoupling oxidative phosphorylation, consequently leading to depletion of parasite ATP [42]. As reported by Braguine et al. [43], it is

possible that different developmental stages (larvae, juvenile or adult) of helminths might possess different susceptibility to selected flavonoids. In recent study performed in 2012 by Zibaei et al. [44] treated parasites revealed complete inactivation and loss of motility/flaccid paralysis that was followed by death at varying periods of time. As a consequence a deformity at the surface architecture of the worms was observed.

The nematotoxicity of ivermectin and levamisole which are conventional pure substances showed some differences with the ethanolic SBE of *A. occidentale* in their mechanism of action. Despite the alleles conferring resistance to *C. elegans* towards levamisole and ivermectin, ethanolic SBE of *A. occidentale* has been lethal to ivermectin- and levamisol-resistant strains. Certainly, ethanolic SBE of *A. occidentale* could have an action on different receptors as conventional drugs.

In order to assess whether the SBEs' mode of action might be similar to levamisol or ivermectin, two mutants have been used. It is known that levamisole and ivermectin are able to increase the membrane conductance and depolarize the membrane by opening non-selective cation ion-channels that are permeable to both Na+ and K+ [45]. Ivermectin is a macrocyclic lactone derivative of avermectin-B isolated from natural source and acts as ɣ-aminobutyric acid (GABA) antagonist in nematodes [45]. It has been extensively investigated that ivermectin acts as GABA antagonists and GluCl channel potentiators [45]. Ivermectin pass through the channels, produce the block at the narrow region and subsequently cause paralysis, immobilization and finally death of worms [46]. In the other hand, levamisole is a nicotinic receptor agonist and causes hypercontraction of muscles and lethality due to prolonged activation of the excitatory nicotinic acetylcholine (nACh) receptors on body wall muscle [47]. Results revealed a varying lethality of the two drug resistant strains to the SBE. CB211 is a knockout mutant of the gene lev-1 which is expressed in body wall muscle and plays a role in egg-laying regulation and normal locomotion. However VC722 is a knockout mutant of the gene glc-2 which represents the binding site of ivermectin in pharyngeal muscle cells. Wild type (LC_{50} 3.17 µg/mL), CB211 (LC_{50} 5.77 µg/mL) and VC722 (LC50 13.21 µg/mL) are all sensitive to SBE after 72 h incubation. This suggests that their efficacy is independent of the levamisole and ivermectin resistance and that the mode of action of the SBE differs from that of levamisole and ivermectin.

The determination of phytochemical constituents in SBE of *A. occidentale*, has revealed the presence of tannins, saponins, flavonoids and phenolic acids. In a study performed by Kubo et al. [48], the same compounds have been found and described as the main active molecules.

Acute toxicity studies on mice showed no death at all doses tested as well as in control group. However, animals treated with extract showed signs of sedation, lethargy and general decreased in physical activity at 1300, 1600 and 1900 mg/kg. In a parallel work, Ofusori et al. [49] reported that *A. occidentale* does not have toxic effect on the brain and kidney of Swiss albino mice. These results indicate that ethanolic SBE given orally under 1900 mg/kg, could be considered relatively safe. Although no mortality has been observed, Okonkwo [50] recorded six mice dead with the bark extract of *A. occidentale* at 1900 mg/kg.

5. CONCLUSION

This work reports that ethanolic stem bark of *A. occidentale* has anthelmintic activities on *O. ochengi* and *C. elegans*. The efficiency of *A. occidentale* extracts can be attributed to the major natural product components present (tannins, flavonoids, saponins and phenolic acids). Further studies have to be carried out to isolate, characterize and elucidate the structures of the bioactive compounds from *A. occidentale* for drug formulation.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This work was carried out in accordance with the Animal Ethical Committee No: 075/16/L/RA/ DREPIA of the Ngaoundere Regional Delegation of Livestock; Fisheries and Animal Indusries, Cameroon.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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