Research Article

In vitro Antifilarial Activities of Ethanolic Extract of the Leaves of *Aloe vera* (l.) Burm. F. (Xanthorrhoeaceae), on the Bovine Parasite *Onchocerca ochengi* and on Drug Resistant Strains of the Free-living Nematode *Caenorhabditis elegans*

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ABSTRACT

Medicinal Plants play an important role in the health care of the world population. It is estimated that more than half of medicinal drugs originates from plants. The filariasis are considered as serious threats to human and animal health. The aim of this work is to assess the antifilarial activities of ethanol extraction in *Aloe vera* leaves on *Onchocerca ochengi* and *Caenorhabditis elegans* and to determine its main phytochemical compounds. The work was conducted at the Laboratory of Applied Zoology a Department of the Biological Sciences of the University of Ngaoundere. Nodules were sampled from the umbilical region of the infected cattle, dissected and the male worms were cultured in RPMI-1640. They were incubated with different concentrations of the plant extracts/reference drugs in RPMI-1640 medium supplemented with penicillin/streptomycin . *C. elegans* was incubated at 18°C with plant extract or drugs in M9-medium and the culture monitored at 48 h and 72 h. The worm viability was assessed biochemically using the dimethylthiazol (MTT) formazan assay. Acute toxicity on *Witar* rats (*Rattus norvegicus*) and phytochemical compounds were determined. Ethanolic extracts of *A. vera* leaves killed *O. ochengi* with dose independent manner with LC_{50} of 21.69 ± 1.19 and 14.49 ± 1.36 µg/mL after 48 and 72 h of incubation. The lowest concentration required to inhibit worms activities (wild-type *C. elegans*) by 50% were 630 ± 0.72 and 500 ± 0.65 µg/mL after 48 and 72 h. That extract contains polyphenols, tannins, flavonoids and saponins. Its respective content was: 1290 ± 0.063; 376.74 ± 0.04; 15.97 ± 0.014 and 0.18 ± 0.0 mg/g. For acute toxicity, no signs of toxicity are recorded at the dose of 3000 mg/kg and there is no rat death. Finally, an interesting antifilarial activity was observed. *A. vera* could then be a potential source of natural antifilarial and anti-onchocerciasis.

Keywords: Antifilarial activities, *Onchocerca ochengi*, *Aloe vera*, acute toxicity.

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INTRODUCTION

Onchocerciasis or river blindness is caused by the filarial worm, *Onchocerca volvulus*, transmitted to humans by a vector agent of the genus *Simulium* [1]. It is the second leading cause of blindness due to to infectious diseases [2]. The adult filarial worms are endoparasites which reproduces and lives in skin nodules for more than fifteen years [3]. The disease is characterized by skin irritations and eyes lesions that may lead to blindness in some cases [4]. The incidence of onchocerciasis has been increasing worldwide, particularly in developing countries. It is estimated that about 123 million of people are in risk and around 37 million infected worldwide, from which 99% living in sub-saharian Africa. Onchocerciasis is responsible for about 270.000 cases of blindness and 500.000 cases of visual impairment [5-6]. Onchocerciasis causes the loss of fertile grounds, for example in Cameroon, about 110.000 km^2 of cultivabled pieces of land were abandoned because of the disease [7]. Consequently, lower agricultural output was observed, in addition to the stigmatization of the people attacks and finally children give up studying to guide the blind adults having river blindness [8]. In Africa, the disease affects 31 nations: out of Nigeria with 25% of all the total infections [9]. In Cameroon, the prevalence of onchocerciasis is estimated at 28% and more than one million people show serious lesions (on skin) [10]. Upon the 178 Health Districts in Cameroon, 108 are endemic with the onchocerciasis [10]. In Ngaoundere as other part of the Adamawa Region, the prevalence of human and animal onchocerciasis is estimated to 30% and 65% respectively [7]. The onchocerciasis was carried to Africa by the African Programme of Fight against Onchocercose (APOC), particularly in West Africa by the Campaign Against Onchocerciasis (OCP) and in America by the Programme of Elimination of Onchocerciasis for America (OEPA) [11]. The control began with the vector control which involved spraying of insecticides and larvicides [12] followed by mass distribution using various combinations of drugs including ivermectin which is a recommended molecule against onchocerciasis chemotherapy [13]. These methods presented had various limitations: resistance of blackflies to insecticides, *O.*

volvulus to ivermectin, and the toxicity of insecticides was observed in the balance ecosystems (example: *Apis melifera*), the secondary effect of ivermectin was marked, this drug has not effect on microfilariae, it has impact on the adult worms [12, 14]. In Africa and Asia, 80% of the population depends on traditional medicine for elementary health care [15].

However, according to the literature, the ethanolic extract of *A. vera* has never been studied on the models of *O. ochengi* and *C. elegans.* Based on current knowledge of the plants, their use in traditional treatment of parasitic diseases, there is an opened possibility for new anthelmintics from medicinal plants. The pharmacological activity studies of *A. vera* revealed that it possesses and acts as an anti-inflammatory, antiviral [16], anti-ulcer, antibacterial [17], anti-fungal and anti-leishmaniasis compound [18-19]. Furthermore *A. vera* was found to prevent infectious diseases by stimulating the activities of the immune system, particularly phagocytic and killing macrophage [20] and T lymphocytes and consequently prevent tumor development [21]. However, despite the traditional use against parasitical diseases, data are lacking on the effects of the plant on onchocerciasis. In the present study, we assessed the effect of the ethanolic extract leaves of *A. vera* on the bovine parasite *O. ochengi* and the free-living nematode *C. elegans*.

MATERIALS AND METHODS

Preparation of plant extract

The extract of *A. vera* leaves were harvested in the town of Ngaoundere, Adamawa Region of Cameroon, (Latitude 7°20'N, Longitude 13°30'E). A sample was identified and authenticated; Voucher specimens have been registered under Number 87406/HNC at the National Herbarium in Yaounde (Cameroon). All chemicals were purchased from Sigma (Deisenhofen, Germany). Ethanolic extracts were prepared according to the method described by Ndjonka *et al* [22]. Briefly, 50 g of powdered plant were put in 500 mL of ethanol-distilled water (70:30 v/v) for 48 h at room temperature, centrifuged (3500×g, 10 min) and filtered over filter papers No. 413 (VWR International, Darmstadt, Germany). The clear filtrate was concentrated by a rotatory evaporator (Buchi Rotavapor R-210, Germany) at 40°C under reduced pressure, and lyophilized. The resulting powder was stored at 4°C for biological test. The plant extracts were diluted with 0.2% dimethylsulphoxide (DMSO) in M9-buffer (1.5 g KH_2PO_4, 3 g Na_2HPO_4, 2.5 g NaCl, 0.5 mL 1 M $MgSO_4$) for *C. elegans* or RPMI-1640 for *O. ochengi* to a final concentration of 100 mg/mL. The solution was mixed thoroughly and stored for biological test activity determination against *O. ochengi* and *C. elegans*.

Isolation and culture of *Onchocerca ochengi* and *Caenorhabditis elegans*

The isolation of *O. ochengi* adult male worms was done as described the method used by Ndjonka *et al* [22]. The fresh pieces of umbilical cattle skin with palpable nodules obtained from the communal slaughter house of Ngaoundere I in the Adamawa Region of Cameroon. Dissection was carried out under dissecting microscope (maximum magnification ×50). Adult worms were isolated and washed in sterile phosphate-buffered saline (PBS). For the antifilarial activity of extracts, the worms were incubated at 37°C test according the method of Borsboom *et al* [23].

The different strains of *C. elegans* were used : N2 Bristol refered to as wild-type (WT) ; the albendazole-resistant mutant strains CB211 (lev-1(e211) IV) ; CB3474 (ben-1(e1880) III) ; VC722 (glc 2(ok1047) I). All strains were obtained from the *Caenorhabditis* Genetic Centre (CGC, Minneapolis, USA). *Caenorhabditis elegans* culture was performed on a solid medium NGM (Nematode Growth Medium). The solid culture medium NGM-Agar was obtained by dissolving in 1000 mL of distilled water 17 g of agar, 3 g of NaCl and 2.5 g peptone from casein, and then autoclaved. 25 mL of 1 M KH_2PO_4/K_2HPO_4; 1 mL of 1 M $MgSO_4$; 1 mL of 1 M $CaCl_2$; 1 mL cholesterol were added prior to use. In the medium was added *Escherichia coli* OP50 solution and 0.5 µL of M9 containing *C. elegans* larvae. The Petri-dish was observed under a microscope to check worm's viability then sealed with a film paper. Those dishes were then incubated at 18°C until obtention of gravid worms prior to the synchronization [22].

Antifilarial screening test

The *in vitro* screening test was performed as described by Cho-Ngwa *et al* [24] and modifications. The screening was performed on *O. ochengi* male worms at increasing concentrations (0 to 40 µg/mL) of plant extracts in RPMI. Positive controls are ivermectin, albendazole and levamisole. The tubes were incubated at 37°C after 48 h or 72 h [17].

The synchronized after chlorox treatment, worms (ten young L4 adults per well) were transferred and incubated from solid medium into 24-well sterile plates containing M9-buffer (each well contains 10 young worms). The cultures of *C. elegans*, increasing concentrations (0-8×10^3 µg/mL) of leaves extracts of *A. vera* were added. Worm mortality rate was determined after 48 h or 72 h at 18°C. Positive controls (ivermectin, levamisole and albendazole) were assessed using the same method (0–20 µg/mL). 0.2% DMSO was used as negative control. Each experiment was conducted in three independent duplicates.

Biochemical determination of worm viability

The effect of the leaves extracts of *A. vera* on adult worms male and *C. elegans* was assess by means of the MTT formazan reduction assay following slight modifications the method described by Comley *et al* [25]. 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. After 72 h of incubation, the treated worms were carefully removed and washed in fresh PBS. Single intact worms 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. Inhibition of formazan formation from MTT directly correlates with worm death. All the MTT tests were done in the dark since the MTT reagent is sensitive to light [26]. LC_{50} values were determined by calculation using Log/probit method [27].

Phytochemical analysis

Preliminary qualitative phytochemical analysis

The qualitative phytochemical analysis is the method based on reactions and colour precipitation. The ethanolic extract of *A. vera* was tested for the presence of secondary metabolites such as tannins, flavonoids, polyphenols and saponins compound according to the standard protocols procedures [28-29].

Quantitative phytochemical test

The dosage of total polyphenols was carried out according to the Folin-Ciocalteu reagent method described by Wong *et al* [30], this method consists of carrying out a series of distilled water and gallic acid: 50 µL of the sample at 0.2 mg/mL are mixed with 200 µL of Na_2CO_3 at 35% (w/w) and 250 µL of the CF reagent diluted to 1/10 (v/v). The mixture was stirred, incubated in the dark at 40°C for 30 min and the absorption is measured at 765 nm using a spectrophotometer (UV-biowave Cambridge, England). The results were presented as mg equivalent of gallic acid per gram of dry materials (mg of GAE/g). Polyphenols quantity was determined by calculation from the standard curve of gallic acid titration.

The quantification of the tannins content of the ethanolic extract of leaves of *A. vera* was carried out using the method described by Wolfe *et al*. [31]. Briefly, 200 µL of the sample were mixed with 35% (w/v) Na_2CO_3 and 100 µL of Folin-Ciocalteu Reagent (FC). The solution was vortexed for one minute, incubated for five minutes and absorption at 640 nm was then measured. The results were expressed in Gallic acid equivalent mg per gram of dry materials (GAE/g).

The total flavonoids determination using the method described by Boizot and Charpentier [33] was measured according to the Aluminium Chloride colorimetric method [34]with some modifications. 0.1 g of each extract to be analyzed is added 2 mL of extraction solvent which consists of 1400 µL of 100% methanol, 500 µL of distilled water and 100 µL of acetic acid. The mixture is filtered using wattman paper in a 25 mL beaker and completed at 10 mL with the extraction solvent. 0.25 mL of this solution is transferred to a 14 mL tube and filled up to 5 mL with the extraction solvent: an analysis solution (Y solution) is obtained. To 1 mL of Y solution 0.2 mL of distilled water and 0.5 mL of $AlCl_3$ were added. The resulting solution was then mixed thoroughly and incubated at room temperature for 1 min. The blanc solution was made of 1 mL Y solution, 0.5 mL distilled water and 0.1 mg/mL rutin. The absorbance was measured at 430 nm using a spectrophotometer. The results are expressed as mg equivalent rutin/g dry materials by referring to the rutin calibration curve.

The saponins content was determined following the modified method described of Junaid *et al* [35]. To 0.1 g of the extract, 1 mL of distilled water was added and vigorously shaken for 30 min. The height of foam was measured by a ruler and quantified as follows: Saponin (mg) = [(0.432) (height of foam in cm after 5 to 10s)+0.008]**/**(weight of sample in gram).

Acute toxicity studies of ethanolic extract of *Aloe vera* in *Wistar* rats

Nine weeks old adult male and female Wistar albinos rats (125.3±0.2 to 241.6±0.5 g) were obtained from the animal facility of the Faculty of Science (FS), University of Ngaoundere (Cameroon). Animal were kept at room temperature 22 ±2°C with a relative humidity of 55±1%, in a room of the Laboratory of Applied Zoology, Department of Biological Sciences (FS), University of Ngaoundere. The experimental protocol was done according to 423 guideline of the Organization of Cooperation and Economic Development (OECD) [36] for chemicals' tests. The animal experience was authorized by the Regional Delegate of Livestock, Fisheries and Animal Industries (N° 075/16/L/RA/DREPIA.

Data Analysis

LC_{50} values were calculated using Log-probit method with SPSS 16.0 software. Data were expressed as mean ± standard error on the mean (M±SEM). Data comparison was done using descriptive statistics (calculation of the averages, mean and percentages of mortalities); analysis of variances (one way-ANOVA) followed by multiple tests of comparison of Turkey. The calculation of the phytochemical metabolites of the plant was performed using standard curve formula y=ax+b, where y is the absorbance and x is the content in mg for g of dry materials. The curves and graphs were plotted using Graph Pad prism 5.10. Values of $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Antifilarial activity of ethanolic extract of *Aloe vera* against *Onchocerca ochengi*

The antifilarial activity of *A. vera* leaves on *O. ochengi* adult males was evaluated in terms of mortality after 48 h and 72 h of incubation. The plant extract induced mortality of *O. ochengi* adult male in a concentration-dependent manner (Figure 1). The leave extract killed, with LC_{50}= 21.69 ± 1.19 and 14.49 ± 1.36 µg/mL respectively after 48 h and 72 h at 37°C (Table 1). Positive controls were active against *O. ochengi* with LC_{50} of 2.23 ± 1.96 µg/mL for ivermectin, 3.62 ± 1.88 µg/mL for levamisole and 4.34 ± 0.71 µg/mL for albendazole after 72 h incubation (Table 1). The ethanolic extract of *A. vera* showed an antifilarial activity similar to ivermectin, levamisole and albendazole after 72 h after post incubation ($P < 0.05$). Our results are superior (LC_{50} values) to those of Ndjonka *et al.*[38], who

evaluated the effect of antifilarial activity of four plants from Nord-Cameroon (stem barks and leaves of *Detarium microcarpum* with LC_{50} of 5 and 7.9 µg/mL) after 72 h incubation ; Kalmobé *et al.* [39], testing in vitro anthelmintic activity of *Lophira lanceolata* (Ochnaceae) on the bovine parasite *O. ochengi* (LC_{50} values of 9.76, 8.05, 6. 39 µg/mL respectively for leaves, trunk bark and root bark after 72 h) and Dikti *et al.*[40], who evaluated the effect of *Acacia nilotica* on *O. ochengi* males (11.5 µg/mL). But, these values are lower than those of Ndjonka *et al.* [41], testing the activity of *Anogeissus leiocarpus* extracts on *O. ochengi* (90 µg/mL after 72 h incubutation); Cho-Ngwa *et al* [24] on *Margaritaria discoidea* and *Homalium africanum* on *O. ochengi* (LC_{50} of 31.25 µg/mL) and Megnigueu *et al.* [37] evaluated on *O. ochengi* (31.01±1.17 µg/mL) after 72 h incubation.

Table 1: LC_{50} of *Aloe vera* crude extracts (ethanolic) and positive control tested against *Onchocerca ochengi* after 48h and 72h exposure.

O. ochengi	**LC_{50} µg/mL after 72h** (after 48h)		
Drugs	**Ivermectin**	**Levamisole**	**Albendazole**
	2.23±1.96^{ns} (5.27±0.01)***	3.62±1.88^{ns} (6.9±0.03)***	4.34±0.71^{ns} (8.0±0.0)***
Leaves	14.49±1.36^{ns} (21.69±1.19)***	20.71±3.14^{ns} 11.75±0.73***	20.71±3.14^{ns} (11.75±0.73)***

Data are mean ±SEM from three independent duplicate experiments. Ns=non-significant (P>0,05); significant with the threshold of 1% (very significant P<0,01, **=) and 1‰ (P<0,001;***= highly significant).

Antifilarial activity of ethanolic extract of *Aloe vera* against *Caenorhabditis elegans*

The LC_{50} values are consigned in the Table 2, a tenfold increase of the LC_{50} value of *C. elegans* albendazole-resistant, ivermectin-resistant and levamisole-resistant mutant CB3474, VC722 and CB211 respectively, was observed at 1573 ± 0,54, 1000 ± 0,91 and 1260 ± 0,07 µg/mL for 72 h respectively for these leaves. Table 2 shows statistical analyses which reveal that, there are no significant statistical difference between mean LC_{50} values of albendazole-resistant strain: CB3474 (1573 ± 0.54 µg/mL); to levamisole: CB211 (1260 ± 0.07 µg/mL) and ivermectin: VC722 (1000 ± 0.91 µg/mL) after 72 h incubation (P>0,05). The LC_{50} values obtained in this work are contradictory to those obtained by Ndouwe *et al* [42], who evaluated the effect of *Anacardium occidentale* on *O. ochengi* and *Caenorhabditis elegans* ivermectin-resistant strain VC722 (LC_{50} 26.62 and 13.21 µg/mL respectively at 48 and 72h) and levamisol CB211 (LC_{50} 10.93 and 5.77 µg/mL respectively, 48 and 72 h) and WT (8.63 ± 0.16 and 3.17 ± 0.18 µg/mL after 48 and 72 h). The differences obtain in their results of one strain to another could be due to the modification of the genes of this nematode into several mutants ones to the conventional drugs. The leaves of *A. vera* may act on the same receptors as ivermectin. The ivermectin-resistant mutant (VC722) is a single mutant in which the GlucI *glc-2* subunit has been mutated. *Glc-2* represents the acceptor of ivermectin in pharyngeal muscle cells [43]. The paralysis and death of the nematode are due to the massive entry of chloride ions (ivermectin effects) into the cells [43]. Levamisole has been proven a nicotinic receptor agonist responsible for muscle hyper-contraction in nematodes. Death of the latter would result from the prolonged excitation of nicotinic muscle receptors [13]. The expression of the *lev-1* allele allows the regulation of locomotion and eggs laying. The *lev-9* allele codes for the synthesis of new extracellular proteins. The mutation of the alleles gives CB211 (lev-1) resistance to levamisole [44].

Qualitative and quantitative determination of secondary metabolites of *Aloe vera* ethanolic extract leaves

Table 3 presents the results of qualitative and quantitative assays. In this table, it appears that the ethanolic extract of *A. vera* content is higher in polyphenols, tannins, flavonoids and saponins. Tannins and polyphenol were the most abundant compound content with 1290 ± 0.063 mg/g and 376.76 ± 0.04 mg/g respectively. Flavonoids were the less quantified content with 15.97 ± 0.014 mg/g and saponine 0.18 ± 0.0 mg/g. The compounds in the extract may act in synergy to kill the parasite. These results are similar to those observed by Arunkumar and Muthuselvam [17], indicated that *Aloe vera* contains flavonoids, tannins and saponins. This result is in accordance with several demonstrating that the tannins, flavonoids and polyphenols are responsible of antimicrobial, antidiarrhoeal, anthelmintic and anticancer activities [17]. The tannins would react directly with surface proteins of the worm according to Massamha *et al* [45]. They cause physiological dysfunctions with regard of the mobility and the absorption of nutrients, leading to the death of worms. Tannins also interfere with energy production in parasitic helminths by decoupling oxidative phosphorylation [46]. The condensed tannins may also bind to the cuticle of larvae which is rich in glycoprotein and cause death suggested by Iqbal *et al* [47].

Acute toxicity test of ethanoloic extract of *Aloe vera*

The result of acute toxicity of ethanoloic extract leaves of *A. vera*, oral administration to the Witar rats showed no mortality to the dose up to 3000 mg/kg and no signs of toxicity after oral administration. The acute toxicity results of A. vera leaves are non-toxic up to this dose (3000 mg/kg) (locomotion, convulsions, loss of appetite and sneezing) were registered. These results are similar to those observed by Michayewicz [16], testing of ethanolic activity of *Aloe vera* leaves and obtaining mortality up to 3000 mg/kg. A similar result was observed by Nghonjuyi et al [48] of leaves *A. vera* (Xanthorrhoeaceae), the acute toxicity test, none of the four studied hydroalcoholic extracts induced mortality or significant behavioral changes.

Table 2: LC_{50} of *Aloe vera* crude extracts and positive control tested against *Caenorhabditis elegans* wild type and ivermectin, levamisole and albendazole mutant resistant strains of the free-living nematode *Caenorhabditis elegans* after 48 h and 72 h post-treatment (Data are mean±SEM from three independent duplicate experiments)

LC_{50} µg/mL after 72 h (after 48 h)				
Strains mutant	**Leaves**	**Ivermectin**	**Levamisole**	**Albenazole**
WT	500±0.65 ^{ns} (630±0.72)***	2.17±0.66 *** (2.41±0.33)***	4.12±0.31*** (4.15±0.68)**	4.26±0.0 *** (4.35±0.57)***
CB3474	1573±0.54 ^{ns} (2617±0.19) **	-	-	> 100
CB211	1260±0.07 ^{ns} (1478±2.10) **	-	> 100	-
VC722	1000±0.91 ^{ns} (1010±8.47) ^{ns}	> 100	-	-

Table 3 : Phytochemical screenings of ethanolic extract of *Aloe vera* leaves

Parts used	**Polyphenols**	**Tannins**	**Flavonoids**	**Saponins**
	(mg/g)			
EtOH (70%)	++	+++	+	+
EtOH (70%)	1290±0,063	376,74±0,04	15,97±0,014	0,18±0,0

+ = present; ++ = very present; +++ = highly present; Data are mean±SEM from three independent duplicate experiments.

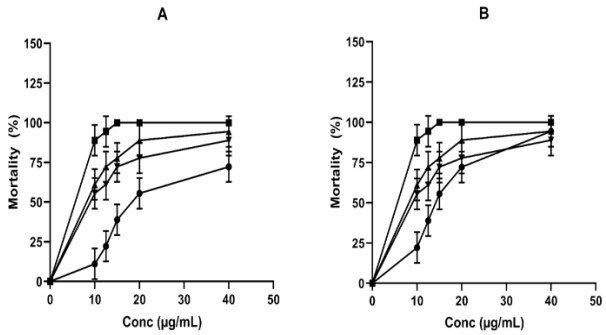

Figure 1: Activity of plant extracts against *Ochengi ochengi* with crude ethanolic extracts from *Aloe vera* leaves (●) Leaves, (■) Ivermectin, (▲) Levamisole and (▼) Albendazole 48h (A) and 72h (B) post-exposure. Data are mean±SEM from three independent duplicate experiments

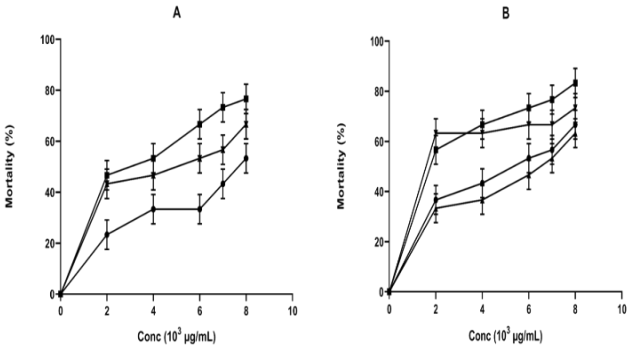

Figure 2: Effects of plant extracts against *Caenorhabditis elegans* wild type and drug resistant strains with ethanolic extracts from *Aloe vera* leaves 48 h (A) and 72 h (B) post-exposure (●) VC722, (■) CB211, (▲) CB3474 and (▼) WT. Data are mean±SEM from three independent duplicate experiments.

CONCLUSION

We assessed the effects of the ethanolic extract of *A. vera* leaves on the *O. ochengi* and free-living nematode *C. elegans* as models for onchocerciasis. The efficiency of *A. vera* extracts can be attributed to the major natural product components present such as polyphenols, tannins, flavonoids and saponins. Further studies have to be carried out to isolate, characterize and elucidate the structures of the bioactive compounds from *A. vera* for *in vivo* antifilarial assay.

Ethics approval and consent to participate

This work was carried out in accordance with the Animal Ethical Committee of the Ngaoundere Regional Delegation of livestock; Fisheries and animal Industries Authority, Cameroon. Gives the number 075/16/L/RA/DREPIA.

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