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Nematocidal Activity on *Onchocerca ochengi*, Toxicity and Phytochemical Screening of *Vernonia perrottetii* Sch. Bip. Ex Walp (Asteraceae) Extracts

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Authors' contributions

This work was carried out in collaboration among all authors. Authors MME and NF designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors NZKS and DNE performed the statistical analysis of the study and managed the literature searches. Authors KFS and ND revised the first draft. All authors read and approved the final manuscript.

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ABSTRACT

Onchocerciasis is a disease caused by a parasitic nematode *Onchocerca volvulus* in human. Ivermectin who is the main drug recommended for the treatment of this disease is only effective against the microfilarial stage of the parasite. Reports of emergence of parasite resistance to ivermectin have complicated onchocerciasis treatment and require to discover novel drugs. The objective of the present study was to investigate in *vitro* anthelmintic properties against the cattle filarial parasite *Onchocerca ochengi*, a model closely related to *Onchocerca volvulus*; and

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evaluate the toxicity *(in vivo)* of local medicinal plant *Vernonia perrottetii.* This plant is used as alternative medicine in the treatment of human onchocerciasis in central and coastal regions of Cameroon. Fifteen crude extracts were prepared from various parts of *V. perrottetii* using three organic solvents (70% ethanol, methanol, methylene chloride) and distilled water. The nematocidal activity was evaluated on adult worms of *O. ochengi*, worm viability was assessed biochemically using the dimethylthiazol (MTT) formazan assay. Oral toxicity of the promising extract was investigated in mice. The ethanolic extracts of the leaves and roots of *V. perrottetii* recorded the highest activities against adult male worms (LC_{50} of 29.80 µg/mL for leaves and 39.36 µg/mL for root). By contrast, the methanol and aqueous extracts of leaves and roots, of the plant as well as the mixture methylene chloride/methanol extracts. For acute treatment, a single dose of 2000 mg/kg no induced critical behavioral changes or death. In sub- acute treatment, daily oral administration of hydro-ethanolic extracts of leaves at the dose of 250, 500 and 750 mg/kg revealed disturbances in the normal growth of animals as well as liver and kidney alterations. Phytochemical analysis of the active extracts revealed the presence of Polyphenols, tannins, saponins and flavonoids. This study revealed the anti-Onchocerca activities of *V. perrottetii*, indicating a possible new source for developing a phytomedicine or drug for the treatment of human onchocerciasis.

Keywords: Onchocerciasis; Onchocerca ochengi; Vernonia perrottetii; oral toxicity; subacute toxicity.

1. INTRODUCTION

Infectious diseases are a permanent challenge to human health worldwide. Bacterial, viral and protozoan infections are common major causes of mortality [1] which are being tackled using various control and prevention tools. However, seventeen infectious diseases are listed as neglected including onchocerciasis [2]. Onchocerciasis, commonly known as a river blindness, is caused by the filarial nematode *Onchocerca volvulus,* and is transmitted by a blackfly vector from the genus *Simulium.* The disease is mainly characterized by skin irritations and eye lesions and can ultimately lead to blindness [3]. It has been estimated that 36 million people are infected [4] and 86 million people live in high risk areas of the African Program for Onchocerciasis Control (APOC) countries. Onchocerciasis is responsible for about 270.000 cases of blindness and 500.000 cases of visual impairment [5]. In the littoral and center regions of Cameroon, the prevalence of human onchocerciasis has been estimated at 50 – 60% [6]. The burden of the disease includes long term disability, social stigmatization and abandonment of the infested areas, which often have a high agricultural [7], leading to economic loss and slowdown of development in the affected country over the years. The two major strategies employed in the control of onchocerciasis include mass treatment of infected people with ivermectin and the elimination of the *Simulium* vector. Despite the successes registered in reducing the disease burden, total elimination has not been achieved due to pitfalls in the control programs. Presently, only ivermectin (Mectizan®, Merck) is recommended for chemotherapy and for mass drug administration. While this drug has marked activity against microfilariae, it has only limited effects on the adult worms. Furthermore, the emergence of ivermectin resistance in parasitic nematodes of veterinary importance [8] raises serious concerns that it may extend to the human *O. volvulus*. Studies have revealed that treatment of some people infected by Onchocerca with ivermectin who are co-infected with *Loa loa* may result in adverse effects, which range from fatigue to consciousness disorders and death [9]. Therefore, novel and complementary helminth control options are urgently needed. Several studies reported the efficacy of plant extracts against helminthes [10]. In order to valorize local plants, our interest was laid on *Vernonia perrottetii* Sch. Bip. ex Walp (Asteraceae). This plant is used in an empirically manner in Cameroonian traditional medicine for the treatment of intestinal worms, malaria and mycosis. In this work, we reported *in vitro* anthelminthic activity of different parts of *V. perottetii* against the *Onchocerca ochengi* filarial parasite.

2. MATERIALS AND METHODS

2.1 Plant Collection and Authentication

The different plants organs (leaves, stem and roots) of *V. perrottetii* were collected in April 2017 from the West Region of Cameroon, following an ethno pharmacological survey.

Preliminary identification was made by botanists at the Department of Biological Sciences, University of Ngaoundere, Cameroon. Authentication was made at the National Herbarium in Yaoundé, Cameroon, where a voucher specimen (14927/HNC) was deposited.

2.2 Preparation of Crude Extracts

All the plant parts collected were air dried for three weeks at room temperature, then ground to fine powder. 10 g of the powdered material were extracted with 100 ml of each solvent for a total of 48 hours at room temperature using five solvents: distilled water (H_2O), 70% ethanol (EtOH), methanol (MeOH), methylene chloride (MC), and a mixture of (MeOH)/ (MC). The solvent was evaporated in a rotary evaporator at appropriate temperature (40°C). After complete removal of solvent, percentage yields were estimated using the following formula: Yield (%) = (Weight of crude extract) × 100/ Weight of powder used. Plant extracts were stored in a refrigerator until use [11]. These extracts were dissolved in dimethyl sulphoxide (DMSO 100%) to prepare a stock solution of 100 mg/mL and kept at + 4°C until used in biological assays.

2.3 *Onchocerca ochengi* Adult Worms Collection

The udders of cattle were collected from the Ngaoundere slaughter house after which they were washed, nodules were removed and kept in phosphate buffer saline (PBS) pH 7.4. The nodules where dissected under a dissecting microscope. Viable male worms present were removed, washed in PBS and rinsed in Roswell Park Memorial Institute medium (RPMI culture medium), as described by Ndjonka et al. [12]. The extraction of female worms was done using collagenase B as described by Simone et al. [13] and Schulz-Key [14] with some modifications. Briefly, nodules extracted from the udder of cattle were incubated in 0.25% collagenase B in RPMI while shaking at 37˚C for 8 hours. Viable female worms were cleaned for removing any remaining tissue, washed with PBS and rinsed in the culture medium. These worms were immediately used for bioassays.

2.4 *In vitro* Assay of *Onchocerca ochengi*

The *in vitro* assay was prepared as described by Cho-Ngwa et al [11] with slight modifications. Firstly, a screening was performed on *O. ochengi* male worms at a single concentration of 300 µg /mL of each crude extract, in order to focus on the most active extracts. The crude extracts showing the best *in vitro* efficiency (100% lethality at 300 µg/mL) were selected for further investigations. For this purpose, adult worms were washed twice and subsequently transferred into RPMI-1640 medium supplemented with L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and incubated at 37ºC in 5% CO_2 atmosphere in 96-well plates (six individuals male worm/ well: 1/100 µl culture medium/well). All tests were done in three independent triplicate determinations. After 72 h exposure, the worms were transferred to fresh PBS to check their lethality followed by the MTT reduction assay.

After wards, extracts that showed 100% activity at primary screens were retested as described under primary screens at concentrations ranging from 0 to 300 µg/mL, this was done to determine their LC_{50} values (lethal concentration of the extract required to kill 50% of worms). During culturing of the female worms, one female worm was each transferred into a well of culture plates (24-well plates) containing 500 µL of the culture medium (RPMI) with different concentrations of plant extract. A simultaneous positive control of Ivermectin (2 mg/mL) and DMSO (dimethyl sulphoxide) was used as negative control. The maximal final concentration of DMSO in negative was 0.015%. Test and control setups were incubated at 37ºC in 5% CO_2 atmosphere during 72 h. Adult worm viability was assessed by the MTT/ formazan assay.

2.5 MTT Reduction Assay

The effect of the plant extract on adult worms (Male and female) was assess by means of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] formazan reduction assay following the method described by Comley et al. [15] with slight modifications. After 72 h of incubation, the treated worms were carefully removed and washed in fresh PBS. Then the parasites were incubated in 500 µL of 0.5 mg/mL MTT solution for 30 minutes. Inhibition of formazan formation from MTT directly correlates with worm death. Treated worms were blotted on absorbent paper and color development of the test compared with the controls. After the MTT assay, worms death are yellow whereas the live worms are blue-purplish.

2.6. Phytochemical Analysis

2.6.1 Preliminary qualitative phytochemical analysis

Vernonia perrottetii (leaves and roots) extracts were subjected to qualitative phytochemical analysis for the presence of various classes of active chemical constituents such as tannins, saponins, flavonoids, alkaloids and phenolic compounds; using standard procedures [16,17].

2.6.2 Quantitative estimation of chemical constituency

Total Polyphenols contents: The total phenolic content within the different extracts was determined by a slightly modified Folin and Ciocalteu method**,** following the protocol applied by Wong et al. [18]. In the procedure, 0.02 mL of the extract, 1.38 mL of distilled water, 0.2 mL of Folin-Ciocalteu reagent mixture and 0.4 mL of 7.5% sodium carbonate were added. The whole was diluted and incubated in the dark. The absorbance was measured at 760 nm. Gallic acid dilutions were used as standard solutions. The results of phenols are expressed in terms of Gallic acid in mg/ml of extract.

Evaluation of tannins: Tannins content were determined by a slightly modified Folin and Ciocalteu method. Briefly, 1 mL of extract was added to 3.75 mL of distilled water and added to 0.25 mL of Folin Phenol reagent and 0.5 ml of 35% sodium carbonate solution. The absorbance was measured at 725 nm. Tannic acid dilutions (0 to 0.5 mg/mL) were used as standard solutions. The results of tannins are expressed

Estimation of flavonoids: Aluminium chloride colorimetric method was used with some modifications to determine flavonoid content. Indeed, 0.2 mL extract, 2 mL of distilled water and 1 mL of 10% Aluminium chloride were mixed and kept at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 430 nm against a blank using the spectrophotometer. Rutin was used as standard (0.1 g/mL); flavonoid contents were determined from the standard curve and were expressed as rutin equivalent (mg/g of extracted compound) [19].

Estimation of Saponins: Test extract was dissolved in 80% methanol, 2 mL of Vanilin in ethanol was added, well mixed and 2 mL of 72% sulphuric acid solution was added, well mixed and heated in a water bath at 60°C for 10 min. Absorbance was measured at 544 nm against reagent blank.

2.7 Toxicity Studies

2.7.1 Oral acute toxicity study

This study was evaluated according to the Organization for Economic Cooperation and Development (OECD) guideline 423 on mice (20-29 g). A highest dose of 2000 mg/kg of extract was used involving three mice. Each mouse was treated with a single oral dose of 2000 mg/kg of extract. Animals were observed individually at least once during the first 30 min after administration, periodically during the first 24 h, and daily thereafter, for a total of 14 days for any clinical signs of toxicity or mortality.

2.7.2 Sub-acute toxicity study

The oral sub-acute toxicity study was carried out according to OECD guideline 407. Adult healthy mice (20-29 g) of each sex were divided into 4 groups of 6 animals (3 males and 3 females per group) each and were placed under standard conditions. Group I was considered as control and the other three groups which were considered as tested groups received the plant extract at a dose of 250, 500 and 750 mg/kg body weight respectively for 28 consecutive days. At the end of the treatment period, the mice were anesthetized with diethyl ether. Blood and organ samples were taken for biochemical analysis. The following serum parameters: glucose, creatinine, Alanine aminotransferase (ALAT), Aspartate aminotransferase (AST) and proteins were tested.

2.8 Column Chromatography Fractionation of the Crude Extract

The extract with the most nematocidal activity from each plant was fractionated. Column chromatography was performed on silica gel 60 (Merck, 70–230 Mesh) with n-hexane, ethyl acetate (EtOAc) and methyl alcohol (MeOH) as eluents. A total of 500 g of dried powder was macerated trice in 1.5 L of EtOH with constant shaking 3 times/day for 3 days. After filtration and evaporation of the combined filtrates, 43 g of the resulting crude ethanol extract was subjected to column chromatography of silica gel using Hexane/EtOAc (1:0–0:1) and EtOAc-MeOH (1:0–5:5) as solvent. Collected fractions were pooled

on the basis of their thin layer chromatography (TLC) profiles [20]. Each fraction was tested for anthelmintic activity against male and female of *O. ochengi*.

2.9 Data Analysis

Lethal concentrations (LC_{50}) and 95% fiducial limits (95% FL) of plant extracts were calculated using the probit analysis and analyzed the difference between treatment groups using one-way ANOVA and LSD in SPSS version 16.1. All results were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1 Yield of Crude Extracts

Table 1 summarizes the percentage recovery of the different extracts of *V. perrottetii*. Higher yield of extract was obtained from etanolic extract of leaves, while the mehtylene chloride of extract of roots showed the lowest percentage

3.2 Nematocidal Activity against Male *Onchocerca ochengi* Adults Worms

Worm motility assay result show that all crude extracts prepared from *V. perrottetii* using solvents of different polarities: distilled water (Aq), 70% ethanol (EthOH), methanol (MeOH), methylene chloride (CH_2CL_2), and mixture (MeOH)/(CH_2CL_2) (v/v); showed decline in motility of the worms at a single concentration of 300 µg/mL after 72 hours of incubation at 37°C. In order to ascertain the degree of mortality in solvent extracts, MTT reduction assay was conducted for the complete set of experiments. The result revealed that on a total of 15 crude extracts prepared from the plant, only 6 crude extracts showed good nematocidal activity against *O. ochengi* male worms; each produced 100% worm lethality at 300 µg/mL. All the plant parts extracts with methylene chloride were inactive on male *O. ochengi* worms, as all stem extract ($LC_{100} > 300$ µg/mL). The active extracts were further screened at various concentrations (0 to 300 µg/mL) on adult male and female worms of *O. ochengi.*

Comparing the mean activities of the different extracts tested against males and females of *O. ochengi* we observed a dose dependent effect from 25-300 µg/mL extract concentration, Mortality increased over time post-exposure and ascending concentrations (Figs 1a and b). Ethanolic extract of leave reached 100% mortality after 72 h incubation at concentrations 100 µg/mL and 150 µg/mL respectively for *O. ochengi* males and females. For the same extract type at a fixed concentration there was a difference in the response of males and females of *O. ochengi*.

The nematocidal activities of each extract showed a dose dependent response for adult worms. LC_{50} values generated by the Log-probit method with SPSS 16.0 software are summarize in Table 1. Ethanolic extracts from the leaves and roots, were the most active against adult worms mortality, with LC_{50} of 29.80 and 39.36 µg/mL respectively for the leaves and roots for male worms; and 40.78 and 52.52 µg/mL respectively for the leaves and roots for female worms; followed by the aqueous extracts, while MeOH and mixture (MeOH)/(CH_2CL_2) displayed very high LC_{50} compared with ivermectin and other extracts.

Compared to ivermectin (LC_{50} values of 42.72 and 46.82 µg/mL respectively for males and females), the 70% ethanolic crude extract of leaves and roots of *V. perrottetii* exhibit macrofilaricidal activity at LC_{50} below that of the positive control ivermectin at the same exposure time (72 h) (Fig. 2)

3.3 Phytochemical Screening Results

The phytochemical screening of the active crude extract of *V. perrottetii* leaves and roots revealed the presence of secondary metabolites such as phenolic compounds, tannins, saponins, flavonoids (Table 2). The quantitative estimation of total polyphenols by the method of folin-Ciocalteu and flavonoids by aluminum trichloride method showed that ethanolic extracts were the richest in polyphenols (6.81 ± 0.08; 4.50 ± 0.08 mg EAG/g extracts; respectively for leaves and roots) and flavonoids (3.36 ± 0.25; 4.33 ± 0.02 mg ER/g extracts for leaves and roots respectively). However, the tannin assay revealed that aqueous extracts yielded the highest value (4.69 ± 0.05 mg EAG/g extracts). Furthermore, estimation of saponin contents by the Vanillin method revealed that, ethanol extracts contains the highest concentration of in saponins (8.57 ± 0.20 mg EV/g for leaves).

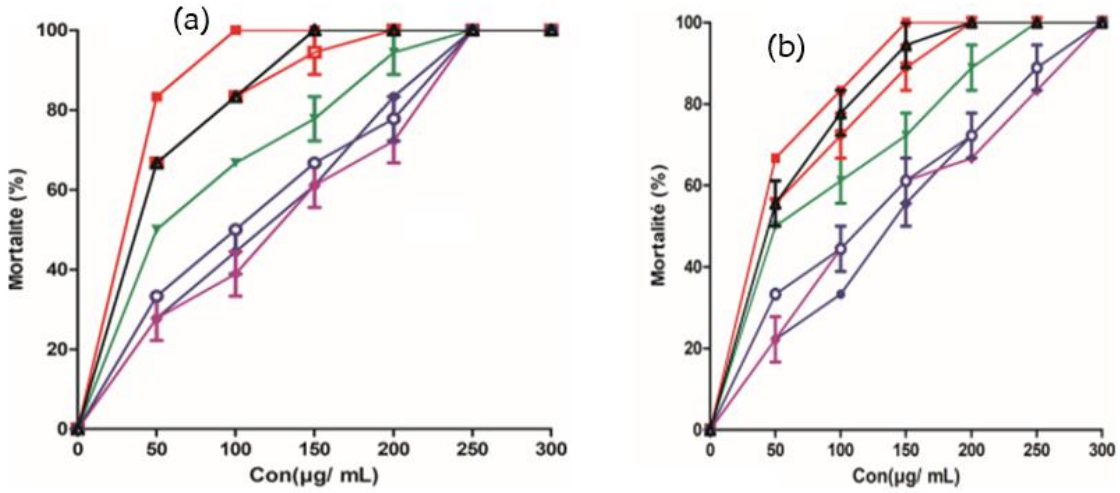

Fig. 1. Mortality of *Onchocerca ochengi* males (a) and females (b) over 72 h when exposed to different concentrations of ivermectin (▲) and crude extracts : Leaves extracts (▼ aqueous, ■EtOH, ●MeOH, ◆MeOH/CH_2CL_2) and roots extracts (■ EtOH, ⊖ MeOH). Data are mean ± SE from three independent triplicate experiments

Table 1. The extraction yield of solvent extracts

	Solvents + yied (%)				
Plant parts	**H_2O**	**EtOH 70%**	**MeOH**	**CH_2CL_2**	**MeOH/ CH_2CL_2 (V/V)**
Leaves	4	**27**	20	5	12
Stems	2	8	8	7	14
Roots	3	8	4	0.5	5

3.4 Acute and Sub-acute Toxicities Study

The acute toxicity test revealed that oral administration of a single dose at 2000 mg/kg of *V. perrottetii* ethanolic leaf extract (VLeth) to six mice separately did not bring out any signs of toxicity or mortality in treated animals during the 14 days observation period. The LD_{50} is greater than 2000 mg / kg, therefore the extract display low toxicity (Class III of the WHO).

In the sub-acute toxicity tests, the effects of daily oral administration at repeated doses of VLeth (250, 500 and 750 mg/kg) to groups of mice were assessed after evaluating the parameters of the biochemical parameters. The results of the biochemical analyses performed are shown in Table 3. All tested doses of VLeth extract induced a significant reduction of glucose in both groups of mice compared to the control groups. However, ASAT and ALAT were statistically different in plant treated group at dose of 750 mg/ kg body weight when compared to mice control group. There was a significant increase in ASAT, ALAT, total proteins and creatinine level ($P< 0.05$).

3.5 Chromatographic Profile and Activity of Fraction on Macrofilariae of *Onchocerca ochengi*

As a result of Colum chromatography, the ethanolic extract of *V. perrottetii* leaves was fractionated into 348 fractions. On the basis of comparative thin layer chromatography studies, 16 combined fractions (F1 to F16) were selected with different Rf values (Table 4). The fractions F1, F6, F7, F8 and F11 seem pure. The anti-onchocerca activity of fractions revealed that, all the fractions exhibited activity against male and female of *O. ochengi*. However, fractions F6, F7, F8 and F11 were the most active. They exhibited lower LC_{50} values than that of the crude extract and the ivermectin. These four fractions were combined in six sub-fractions (A to F) and bioassay results reveal that sub fraction D was the most active with LC_{50} of 9.2 ± 0.3 µg/mL for male (Table 4).

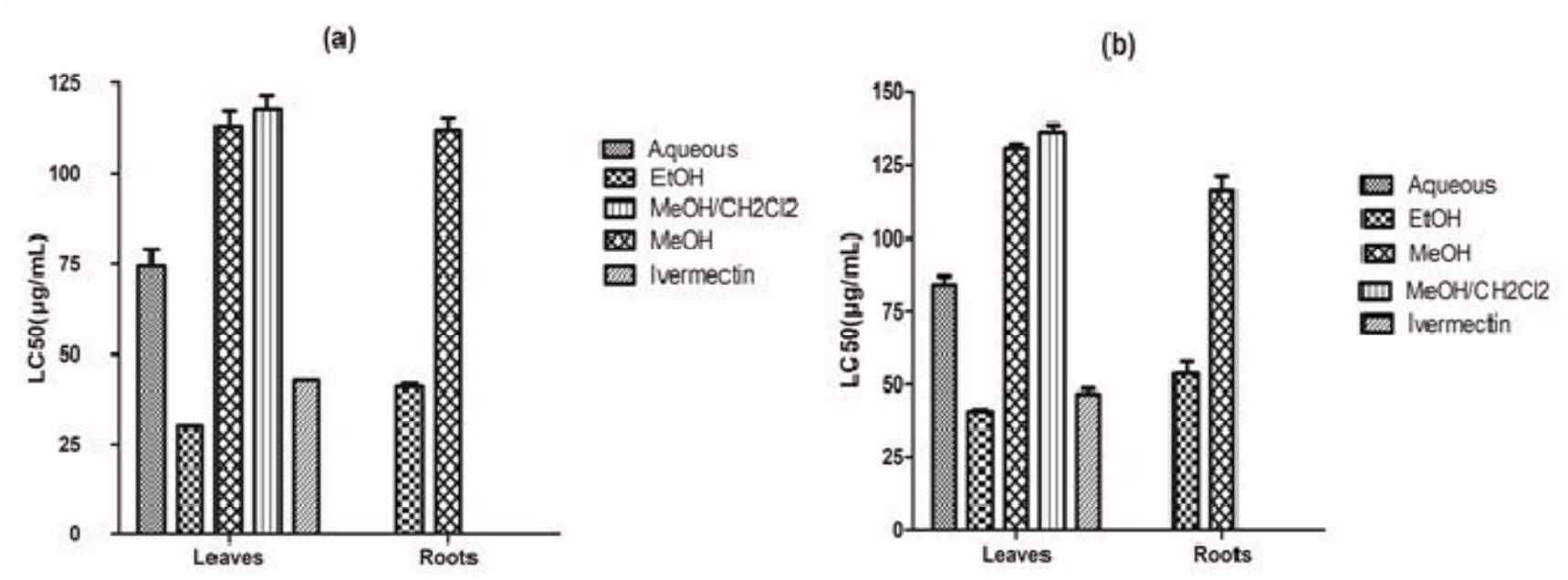

Fig. 2. Comparison of LC_{50} of *Onchocerca ochengi* males (a) and females (b) exposed to different crude extracts and to the values for ivermectin

Table 2. LC_{50} values of different crude extracts on the mean activity of *Onchocerca ochengi* males and females

LC_{50} ± SD (µg /mL) after 72h							
Plant parts	**Leaves**				**Roots**		**Control**
Solvents	**Aqueous**	**EtOH (70%)**	**MeOH**	**MeOH/CH_2CL_2**	**EtOH (70%)**	**MeOH**	**Ivermectin**
Males	74.40 ± 4.85^{c}	29.80 ± 0.00^{bc}	113.18 ± 4.01^{ac}	117.65 ± 3.99^{ac}	39.36 ± 0.60^{abc}	112.13 ± 2.92^{ac}	42.78 ± 0.00^{abc}
Females	84.27 ± 2.77^{c}	40.78 ± 0.57^{ac}	130.63 ± 1.41^{abc}	136.21 ± 2.46^{ac}	52.52 ± 0.77^{ac}	116.10 ± 4.93b^{c}	46.82 ± 1.85^{ac}

a,b,c,: Mean ± SD (Standard deviation) values with the same letter for the different extracts are not significantly different at $p < 0.05$. Values with letter (b) for the different extracts are significantly different at $p < 0.05$. Values with letter (c) for the different extracts are significantly different by the SLD test at $p < 0.001$

Table 3. Total phenolic, flavonoid, tannins and saponins contents for different active extract of *Vernonia perrottetii*

Plants parts extracts	Polyphenol (mgGAE/g)	Tannins (mgGAE/g)	Flavonoids (mgER/g)	Saponins (mgEV/g)
VLaq	3.61 ± 0.21	4.69 ± 0.05	1.33 ± 0.1	0.93 ± 0.00
VLeth	6.81 ± 0.08	2.74 ± 0.04	3.36 ± 0.25	8.57 ± 0.2
VLmet	0.91 ± 0.3	0.6 ± 0.07	2.04 ± 0.04	0.98 ± 0.01
VLmet/mc	1.6 ± 0.3	0.93 ± 0.00	1.04± 0.14	1.18 ± 0.04
VReth	4.57 ± 0.08	1.07 ± 0.02	4.33 ± 0.02	0.0 ± 0.00
VRmet	2.25 ± 0.2	1.28 ± 0.1	2.56 ± 0.4	0.0 ± 0.00

ER: rutin equivalent, GAE: Gallic acid equivalent, EV: vanillin equivalent, VL: Vernonia leaves, VR: Vernonia roots

Table 4. Biochemical parameters of males and females mice after 28 days of treatment with ethanolic extract *Vernonia perrottetii* leaves

Parameter	Control		250 mg/kg		500 mg/kg		750 mg/kg	
	Male	Female	Male	Female	Male	Female	Male	Female
ALT (Ul/l)	21.48 ± 8.02	23.28 ± 2.09	12.22 ± 2.66	22.11± 9.14	29.1 ± 15.3	39.57 ± 24.5	34.33±10.2**	44.81 ± 57**
AST (Ul/l)	43.73± 20.15	62.85 ± 9.6	28.51 ± 11.5	20.95±15.7	52.96 ± 19.6	36.66 ± 23.05	73.28±45.5**	94.2 ± 37.3**
Protein (g/dl)	5.11 ± 0.02	5.5 ± 0.02	3.83 ± 0.01*	3.61 ± 0.01	4.57 ± 0.02*	3.69 ± 0.01	4.25 ± 0.02*	4.04 ± 0.02*
Creatinine (mg/dl)	0.23 ± 0.2	0.24 ± 0.23	0.35 ± 0.2	0.32 ± 0.24	0.47 ± 0.2	0.38 ± 0.37*	0.23 ± 0.2	0.28 ± 0.23*
Glucose (mg/dl)	456 ± 0.3	456 ± 0.3	328.69 ± 0.1**	227.4 ± 0.2**	279.84 ± 0.00**	238.37 ± 0.2**	397.29 ± 0.0**	217.44 ± 0.2**

*Values are means±SEM, n= 3, **P<0.05, a significant difference compared to the control. SEM: Standard error of the mean. ALT: Alanine transaminase, AST: Aspartate aminotransferase*

Table 5. Chromatographic profile and activity of fractions on macrofilariae of *Onchocerca ochengi*

Solvent system			LC_{50} ± SD (µg /mL) after 72h	
Hex : EtOAc	**Fractions**	**Rf value**	**Males**	**Females**
100 : 00	1-14 : F1	0.66	37.26 ± 0.2	44.99 ± 0.3
90 :10	15-26 : F2	0.86; 0.69; 0.51	63.66 ± 0.3	101.66 ± 0.1
80 : 20	27 - 45 : F3	0.82 ; 0.51	54.00 ± 0.3	124.00 ± 0.3
70 : 30	46 - 60 : F4	0.85; 0.70; 0.55	71.11 ± 0.2	141.11 ± 0.1
60 : 40	61 - 81 : F5	0.65; 0.57; 0.43	73.41 ± 0.3	143.41 ± 0.2
50 : 50	**82 - 103 : F6**	**0.56**	**23.34 ± 0.3**	**32.34 ± 0.1**
40 : 60	**104 - 120 : F7**	**0.59**	**21.26 ± 0.2**	**20.92 ± 0.0**
30 : 70	**121 - 145 : F8**	**0.55**	**22.33 ± 0.1**	**31.66 ± 0.1**
20 : 80	146 - 160 : F9	0.69 ; 0.51	31.74 ± 0.1	72.74 ± 0.2
10 : 90	161 - 187 : F10	0.68 ; 0.51	30.95 ± 0.2	81.04 ± 0.1
100 : 00	**188 - 215 : F11**	**0.43**	**21.44 ± 0.1**	**31.44 ± 0.1**
EtOAc : MeOH				
90 : 10	216 - 236 : F12	0.59; 0.43	49.54 ± 0.1	139.54 ± 0.2
80 : 20	237 - 256 : F13	0.85 ; 0.69 ; 0.20	80.35 ± 0.3	143.69 ± 0.2
70 : 30	257 - 280 : F14	0.85 ; 0.69 ; 0.20	73.69 ± 0.4	133.69 ± 0.2
60 : 40	280 - 300 : F15	0.66 ; 0.20	36.11 ± 0.3	116.11 ± 0.1
50 :50	301 - 348 : F16	0.66 ; 0.21	35.44 ± 0.1	115. 44 ± 0.1
Combinations of most active fractions				
	F6 + F7 : A	0.23	12.5 ± 0.3	Nd
	F6 + F8 : B	0.25	10.3 ± 0.3	Nd
	F6 + F11 : C	0.40; 0.24	16. 4 ± 0.3	Nd
	F7 + F8 : D	**0.21**	**9.2 ± 0.3**	Nd
	F7 + F11 : E	0.32 ; 0.26	17.1 ± 0.3	Nd
	F8 + F11 : F	0.27 ; 0.20	14.7 ± 0.3	Nd

Each value represents mean ± SD (standard deviation); Nd: not determined; Hex: hexane; EtOAc: ethyl acetate; MeOH: methyl alcohol; Rf: Retention factor

4. DISCUSSION

The use of nematicidal bio-products obtained from plants is an alternative control method that could partially replace the use of chemical anthelmintic drugs against cattle and sheep parasites. This is the first attempt at using *V. perrottetii* against *O. ochengi*. This study evaluated the anthelmintic activity of *V. perrottetii* against *O. ochengi* males and females. The different secondary metabolites that are probably responsible of the anthelmintic activity of *V. perrottetii* were also determined and then the potential toxicity of the ethanolic extract of the leaves of *V. perrottetii* in acute and sub-chronic oral administration in rodents were evaluated.

Dose-dependent activity relationships were observed with the six extracts for *O. ochengi* male and female (Fig. 1). This indicates high anti-onchocerca properties of the plant extracts. Previous studies have shown that some Cameroonian medicinal plants, including *Acacia nilotica*, *Cucurbita pepo ovifera var ovifera*, *Anacardium occidentale*, *Lantana camara, Tamarindus indica*, and *Milletia comosa* also have anti *O. ochengi* activity [21-25]. The demonstration of such good activity by this plant has come to expand the list.

The median lethal concentration (LC_{50}) of all crude extracts of *V. perrottetii* varied with solvent used in extraction of active compound (Table 1). Encouraging results were achieved with *V. perrottetii* ethanolic extract of leaves, showing significant differences against male and female worms when compared with the effect produced by the standard drug ivermectin and all the extracts. This could probably be related to the different chemical compound extracted in the different solvents and their biological effects on parasite. The ethanolic extract of leaves was more active against adult males (LC_{50} of 29.80 µg/mL) than adult females (LC_{50} of 40.78 µg/mL) (Figs.1a and b); These differences of a particular extract tested at a single concentration acting differently on the different genus of parasite might be due to the differences in proteins being expressed at the different stages or species.

Anthelmintic drugs are known to act by causing paralysis of the worm, or damaging cuticle, leading to partial digestion or to rejection by immune mechanism. Anthelmintic drugs also interfere with the metabolism of worm, and since the metabolic requirement of these parasites vary greatly from one species to another [26]. The pharmacological potential of plants is attributed to the presence of a wide array of phytochemical compounds in them [27-28]. Therefore, in the current study an effort was made to explore potential active compounds from the studied plant extracts. The phytochemical screening of the six promising extracts revealed the presence of polyphenols, flavanoids, tannins and saponins; but with different content depending on the extraction solvents (Table 2); suggesting that active principles in the extracts may be from the afore mentioned groups of compounds. Chemically, tannins are polyphenolic compounds. Earlier studies reported that flavonoids present in plants extracts could be the reason for paralysis of worms [29-30] and some synthetic phenolic compound lie niclosamide, oxyclozanide and bithionol could pharmacolocally interfer with energy generation in helminth parasites by uncoupling oxidative phosphorylation reaction [31]. It could be possible that tannins contained in our extracts produced similar effects. Another study stated that tanins might display it anthelmintic effect by binding with free proteins in the gastrointestinal tract of the host animal or the parasites cuticle and cause death [32]. From the results of our analysis, there is moderately high amount of tanin in our aqueous extracts compared to ethanol extracts; a high significant anthelmintic activity of VLeth could be due to saponins or synergy among the different bioactive constituents. The main biologic activity ascribed to saponins based on recent research is their membrane permeability property. In fact, saponins interact with the sterols of the parasitic worms cell membrane, provocatively increasing membrane permeability and cell death [33]. This cytotoxicity mechanism can be at the origin of the anthelmintic action of VLeth. It appears that this plant is a source of polyphenol, flavonoids tannins and saponins. The highest content is that of the ethanolic extract and we can therefore say that 70% ethanol is the best solvent among all those used.

After confirming the nematocidal activity of VLeth, it was deemed necessary to investigate and obtain data on their safety. Therefore, an evaluation of the acute and subacute oral toxicities of the most effective extract (VLeth) was carried out. Several parameters were evaluated after the *in vivo* acute and sub-acute oral administration of VLeth. Mortality is an important criterion on toxicological evaluation [34] and both acute and sub- acute administration of the extract did not induce a significant mortality. In the acute study, no death animals were recorded within 14 days after acute oral administration of the extract. The LD_{50} of the extract was estimated to be more than 2000 mg/kg via the oral route. According to the globally Harmonized Classification system for chemical substances and mixtures (GSH) adopted by the OECD, the plant extract could be assigned as a class 3 drug and then, considered as a weakly toxic substance [35].

Sub-acute toxicity study is essential in assessing the bioaccumulative effects of xenobiotics in biological systems. Assessment of liver and kidney function is very important in evaluating toxicity of modern and traditional medicines since these organs play major roles in metabolism of xenobiotics in the body [36]. In the sub-acute toxicity evaluation, only one male animal treated with a dose of 500 mg/kg died. However, the results showed no dose-response relationship and mortality was not clearly related with extract administration since deaths occurred only with lower doses, indicating that they might have another cause like an improper gavage. The liver is a vital organ that plays a central role in drug biotransformation, and its normal function is assessed by various serum biomarker enzymes [37]. Liver damage are usually assessed by the determination of serum transaminases (ALT and AST) and also by the measurement of total proteins. Transaminases are distinguished indicators of liver function and used as biomarkers to conclude the possible toxicity of drugs and xenobiotics [38]. Increased ALT levels in the serum reflect hypertrophy and injury in liver tissues [39]. The AST level, apart from being an indicator of liver dysfunction, is also used to assess muscle and heart diseases [38]. Regarding the hepatic function evaluation, the VLeth extract produced significant changes ($P \leq 0.05$) in serum levels of ALT and AST, since significant increase in the levels of these parameters was noted in VLeth treated groups compared to the controls. This may suggest apparent hepatotoxicity effect in mice. Interestingly, a significant decrease in the level of ALT and AST was observed in the female and male group treated with 250 mg/kg of VLeth extract, which suggest that the extract may

exhibit a potential hepato-protective effect at this dose. Serum total protein levels is a rough measure of protein status that can reflect major functional variations in kidney and liver functions. Abnormal levels of may be associated with liver infections or chronic inflammation [40]. In our study, there were no significant variations in the levels of serum total protein within the treated groups relative to the control groups. This indicates that VLeth did not cause any damage to the liver.

The sub-acute toxicity of the extracts on kidney function was evaluated by using serum creatinine, as marker. In this study, no significant differences ($P > 0.05$) in the level of creatinine was noted among VLeth treated groups in comparison to the control group. These findings may indicate that the extract at the doses tested did not induce alterations in renal function or kidney damage. Further, there was a low level of glucose in the treated group as compared to the control group which may be due to inadequate insulin secretion that indicates normal functioning of the liver.

Phytochemical studies showed that the VLeth extract contains various secondary metabolites such as polyphenols, saponins, tannins, and flavonoids. These bioactive constituents present in the extract may account for the observed pharmacological effects, as suggested by Ntchapda et al. [41] when studying the subacute toxicity of leaves of *Ficus glumosa* in rodents. Several studies have reported that polyphenols and flavonoids compounds bring their hypoglycemic effect through different mechanisms, including inhibition of α-amylase and α-glucosidase activities, enhancement of peripheral glucose uptake, and stimulation of insulin secretion from pancreatic β cells [42].

The fractionation of hydro-ethanolic extract resulted in 384 samples were collected and grouped according to the eluents in16 fractions. The anthelmintic bioassay test of showed significant differences in the activity of the different fractions against adults worms. This probably means that several compound are responsible for the activity. The identification of small LC_{50} values by F6, F7, F8 and F11 amongst many fractions, when compared with the standard drug, ivermectin and crude extract in Table 4 shows that the secondary metabolites or active compounds responsible for the anthelmintic properties may be concentrated in these fractions. These four fractions were combined in six sub-fractions (A to F) and bioassay results reveal that sub fraction D was the most active with LC_{50} of 9.2 ± 0.3 µg/mL for male (Table 4). This implies that active principles from this extract may be unstable, unable to withstand the fractionation process; or may be acting in synergy to provide the anti-parasitic activity, or even might have been over retarded in the column or missed out in the chromatographic process.

5. CONCLUSION

In order to assess the nematocidal properties of *Vernonia perrottetii*, a total of 15 extracts were prepared from that plant and screened *in vitro* against *Onchocerca ochengi* parasite. Acute and sub-acute oral toxicity of active extract were tested on mice. Overall, results from this study suggests that crude extracts from solvents of different polarities using *V. perrottetii* exhibit selective filaricidal activity and could serve as potential sources of new drugs against onchocerciasis. It also validates the traditional use of this plant in local treatment of onchocerciasis.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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