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# ***In vitro* Anthelmintic Activities of Extracts and Fractions of *Cosmos sulphureus* Cav, Against *Onchocerca ochengi***

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**Abstract:** Medicinal plants have been of great importance to many traditional communities for many generations. However, there is need to carry out scientific studies in order to confirm the medicinal properties of many plants used traditionally. *Cosmos sulphureus* (Asteraceae) used by local communities for the treatment of various diseases has showed antioxidant, antibacterial, antifungal and antiplasmodial properties although there are no studies demonstrating its antionchocerca activity. The present study was undertaken to investigate the antionchocerca potential of crude extracts and chromatographic fractions of *C. sulphureus* using *Onchocerca ochengi*, a bovine filarial closest in phylogeny to *Onchocerca volvulus*. Solvent extraction of the parts of *C. sulphureus* was performed using distilled water, 70% EtOH, MeOH, CH<sub>2</sub>Cl<sub>2</sub> and a mixture of MeOH/CH<sub>2</sub>Cl<sub>2</sub> (v/v). Anthelmintic assay was evaluated on adult worms of *O. ochengi* and worm viability was assessed biochemically using the dimethylthiazol (MTT) formazan assay. Acute and sub-acute oral toxicities of the promising extract was investigated in mice. The chemical composition of extracts was revealed. EtOH extract of roots showed highest anthelmintic activity with an LC<sub>50</sub> value of 31.01±1.17 µg/mL which was more significant than the one of ivermectin (LC<sub>50</sub>=42.78 µg/mL) used as standard. The other extracts show moderate activities. The most active fraction obtained from EtOH extract of roots had an LC<sub>50</sub> value of 19.10 µg/mL on male worm. For acute toxicity, a single dose of 2000 mg/kg used induced no critical behavioral changes or death. In sub-acute toxicity, daily oral administration of hydro-ethanolic extracts of roots 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. These results unfold potential sources of novel anti-onchocerca lead compounds and validate the traditional use of the plants in onchocerciasis treatment.

**Keywords:** Anthelmintic Activity, *Onchocerca ochengi*, *Cosmos sulphureus*, Acute and Sub-acute Oral Toxicity, Phytochemicals

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## **1. Introduction**

Onchocerciasis, well known as river blindness, is a debilitating insect-borne parasitic disease caused by *Onchocerca volvulus* and transmitted to human by Simulium species (black flies) that breed in fast flowing rivers and streams. The adult filarial worms reproduce and live in skin

nodules of human body for long period of time, even for more than fifteen years [1]. Black flies of Simulium species, especially, *Simulium damnosum*, is the vector and reservoir for onchocercal microfilarial [2]. The disease is endemic in 37 countries in West, East and Central Africa, the Arabian Peninsula and parts of South and Central America [3]. According to Global Burden of Disease Study, there were

20.9 million prevalent *O. volvulus* infections worldwide in 2017; 14.6 million of the infected people had skin disease and 1.15 million had vision loss. More than 99% of infected people live in 31 African countries. Adult *O. volvulus* average lifespan is estimated to 10-15 years [4, 5]. Individual female worms produce daily thousands of microfilariae whose lifespan varies from 12 to 18 months. By invading the host dermis and eyes, live microfilariae interact with the host immune system, while dead microfilariae induce inflammatory responses, causing a variety of skin and ocular symptoms [6, 7]. Infection leads to severe skin damage with unrelenting itching, visual impairment and blindness. Irreversible onchocercal blindness is ranked as the world's second leading infectious cause of preventable blindness after trachoma [8]. Besides its clinical impact, river blindness also has an important socio-economic impact on affected populations. It creates stigma and generates and perpetuates poverty [9]. Fear of the disease often prompted people to abandon fertile lands which in turn led to an increase in poverty and famine, making the disease a major obstacle to socioeconomic development. The agricultural productivity is therefore hindered, generating massive economic losses and imposing a disproportionate disease burden in poor rural communities [10]. Current efforts to control onchocerciasis are almost exclusively dependent on controlling transmission using mass distribution of ivermectin. This drug is unfortunately, only a microfilaricide, and a major challenge with this treatment is the fact that it also kills *Loa loa* mf in blood, a situation that often leads to severe adverse effects (encephalopathy and death) in individuals with high *L. loa* mf load [11]. In addition, there is evidence of resistance or low response rate of mf to ivermectin [12, 13]. Therefore, there is a need for a safe and effective macrofilaricidal drug against onchocerciasis that will be able to cure the infection and break transmission cycles, or at least, an alternative microfilaricide that does not kill *L. loa* mf.

A number of plant species have been claimed by folklore as treatment for various diseases, including onchocerciasis. *Cosmos sulphureus*, one of them used traditionally as anthelmintic in Cameroon. It is a plant belonging to the Asteraceae family, commonly known as yellow cosmos spread throughout India, Florida, Southern United States and South America [14]. This plant is generally used for improving blood circulation, antiaging agent, reducing body heat, strengthening bone marrow and to treat infections associated with pathogenic microorganisms [15]. *C. sulphureus* have a long traditional use in Brazil and Mexico for the treatment of malaria [16]. In Cameroon, infusions and decoctions of its parts are commonly prescribed as a remedy for helminth infections and malaria. It has been reported that the acetone extract of leaves of *C. sulphureus* reduce the hatching rate of eggs and inhibit the motility of adults *Haemonchus contortus* [17]. Studies by Kayser *et al*, carried out in 2011, revealed an antiparasitic activity of *C. sulphureus* against Leishmania species and *Plasmodium falciparum*. Studies on the chemical composition and biological activity of *C. sulphureus* suggest that leaves and

roots of the plant have anti-plasmodial, antibacterial, anti-fungal, as well as antioxidant activities [18, 19, 20]. Moreover, there is no information concerning to the antionchocercal potential of *C. sulphureus*. Based on the traditional knowledge of medicinal system, the present study was carried out to evaluate the anti onchocercal activity of some extracts of leaves and root of *C. sulphureus*. In its local use, the leaves and roots of the plant are chopped, dried, boiled in water and taken as a decoction. The activity was evaluated on the adult worms of *Onchocerca ochengi*, the closest relative of *O. volvulus* and best model for anti-onchocerciasis drug screens [21].

## 2. Materials and Methods

### 2.1. Collection and Identification of Plant Material

*Cosmos sulphureus* cav were collected in July 2017 from the West Region of Cameroon, following an ethno botanical 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 (20463/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. Ten grams of the powdered material were extracted with 100 mL of each of the following solvents for a total of 48 hours at room temperature: distilled water (H<sub>2</sub>O), 70% ethanol (EtOH), methanol (MeOH), methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>), and a mixture of (MeOH)/ (CH<sub>2</sub>Cl<sub>2</sub>). The solvent was evaporated in a rotary evaporator at appropriate temperature. After complete removal of solvent, percentage yields were estimated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of crude extract}}{\text{Weight of powder used}} \times 100$$

Plant extracts were stored in a refrigerator until use. 0.3g of each extract was weighed and dissolved in 150 mL of 100 % Dimethyl sulfoxide (DMSO) to make a stock concentration of 100 mg/mL from which the various concentrations used were obtained [22].

### 2.3. Phytochemical Analysis

#### 2.3.1. Preliminary Qualitative Phytochemical Analysis

The obtained *C. sulphureus* (leaves and roots) extracts were subjected to qualitative phytochemical analysis for the presence of various classes of chemical constituents such as tannins, saponins, flavonoids, alkaloids and phenolic compounds; using standard procedures [23, 24].

#### 2.3.2. Quantitative Phytochemical Study

Total Polyphenolic contents (TPC): The total phenolic content of extracts of *C. sulphureus* was evaluated by the Folin-Ciocalteu reagent method describe by Wong *et al* [25],

with slight modifications. Indeed, 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% Na<sub>2</sub>CO<sub>3</sub> were mixed and incubated in the dark. The absorbance was measured at 760 nm. Gallic acid was used as standard solutions. The results were expressed in terms of Gallic acid in mg/mL of extract. All the experiments were carried out in triplicate.

Evaluation of tannins (TTC): The amount of condensed tannins in different crude extracts were estimated by a slightly modified Folin -Ciocalteu method. Briefly, 1 mL of extract was added to 3.75 mL of distilled water along with 0.25 mL of Folin reagent and 0.5 mL of Na<sub>2</sub>CO<sub>3</sub> 35% solution. The absorbance was measured at 725 nm. Tannic acid was used as standard.

Total flavonoid content (TFC): TFC was measured according to the Aluminium Chloride colorimetric method [26] with some modifications. Indeed, 0.2 mL of extract, 2 mL of distilled water and 1 mL of AlCl<sub>3</sub> 10% 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 (0.1g/mL); TFC were determined from the standard curve and were expressed as rutin equivalent (mg/g of extracted compound). All experiments were carried out in triplicate.

Total Saponins content (TSC): Test extract was dissolved in 80% MeOH, 2 mL of Vanillin in EtOH was added, well mixed and 2 mL of H<sub>2</sub>SO<sub>4</sub> 72% 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.

#### 2.4. Chromatography Fractionation of the Most active Extract

The most active extract was fractionated. Column chromatography (CC) was performed on silica gel 60 (Merck, 70-230 Mesh) with hexane (Hex), ethyl acetate (EtOAc) and methanol (MeOH) as eluents. A crude extract of 43 g was impregnated with an equal mass of adsorbent. The mixture was subjected to CC with a gradient of hexane (Hex-EtOAc [0-90%]), followed with a gradient of ethyl acetate (EtOAc - MeOH [0-50%]). Collected fractions were pooled on the basis of their thin layer chromatographic profiles [24].

#### 2.5. Isolation of *Onchocerca ochengi* Adult Worms

The isolation of *O. ochengi* adult male worms was done as described previously by [27] from fresh pieces of umbilical cattle skin with palpable nodules obtained from the communal slaughter house of Ngaoundere in the Adamawa region of Cameroon. Nodules removed from the skin were washed, drained and sterilized with EtOH 70% for dissection. After that, *O. ochengi* were extracted, isolated and washed three times in sterile phosphate-buffered saline (PBS). For the anthelmintic activity of extracts, the worms were incubated at 37°C. The extraction of female worms was done using collagenase B as described by Simone *et al* [28] and Schulz-Key [29] 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.

#### 2.6. *In vitro* Assay of *Onchocerca ochengi*

The *in vitro* assay was performed as described by Cho-Ngwa *et al* [30] with slight modifications. Firstly, a screening was performed on *O. ochengi* male worms at a single concentration of 300µg /mL of each crude extract. The most active crude extracts 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 37 °C in 5% CO<sub>2</sub> atmosphere in 96-well plates (six individuals male worms/well: 1/100µL 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 screening were retested as described under primary screening at concentrations ranging from 0 to 300 µg/mL, this was done to determine their LC<sub>50</sub> values (lethal concentration of the extract required to kill 50% of worms). During female worms culture, 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 control was 0.015%. Test and control setups were incubated at 37°C in 5% CO<sub>2</sub> atmosphere during 72 h. Adult worms viability was assessed by the MTT/formazan assay.

#### 2.7. MTT Reduction Assay

The effect of the plant extracts on adult worms (Male and female) was assess by means of the MTT formazan reduction assay following the method described by Comley *et al* [31] 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, worm's death are yellow whereas the live worms are blue-purplish.

#### 2.8. Toxicity Studies

##### 2.8.1. Oral Acute Toxicity Study

The acute toxicity studies were conducted as per the OECD guidelines 420 [32], where the limit dose of 2000 mg/kg body weight used. Observations were made and recorded systematically after 1, 2, 4 and 24h of

administration dose for skin changes, morbidity and aggressiveness, sensitivity of the sound and pain as well as respiratory movement.

### 2.8.2. Sub-acute Toxicity Study

The oral sub-acute toxicity study was also carried out according to OECD guideline 407. Adult healthy mice (20-29 g) of each sex were divided into 4 groups of 6 (3 males and 3 females per group) and 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, 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.

### 2.9. Data Analysis

All experiments were done in triplicate. Statistical analysis of the differences between mean values obtained from the test extracts compared to the controls was done using one-way ANOVA and LSD in SPSS version 16.1. A probability value of  $p \leq 0.05$  was considered to be statistically

significant. The custom Log-probit analysis with SPSS 16.0 software was used to determine lethal concentrations 50 (LC<sub>50</sub>).

## 3. Results and 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 animal's parasites. This is the first attempt at using *C. sulphureus* against *O. ochengi*. The utilization of different extraction methods or even the simultaneous use to the plant extract could be evaluated in order to get the best results.

### 3.1. Yield of Crude Extracts

A total of 15 extracts were prepared from different parts of *C. sulphureus* using solvents of different polarities: methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>) followed by mixture (MeOH) / (CH<sub>2</sub>Cl<sub>2</sub>) (v/v), ethanol 70% (EtOH), methanol and then distilled water (H<sub>2</sub>O). Generally, the percentage yields of ethanolic extracts were highest while those of methylene chloride were lowest. According to the plant part the highest average of yield extractions came from leaves (Table 1).

Table 1. Plant parts and yield of crude extracts obtained with the different solvents.

Plants parts	Solvents + yield (%)				
	H <sub>2</sub> O	EtOH 70%	MeOH	CH <sub>2</sub> Cl <sub>2</sub>	MeOH/CH <sub>2</sub> Cl <sub>2</sub> (v/v)
Leaves	9	18	11	0,8	8
Stems	4	10	6	2	9
Roots	3	4	2	0,4	3

Since the different extractions were carried out under the same conditions, the combined use of water and organic solvent would have facilitated the extraction of chemicals that are soluble in water and / or in the organic solvent. This could therefore justify the high yield we obtained with the aqueous ethanol solvent (70%).

### 3.2. Phytochemical Analysis

The results of the phytochemical Screening of the Plant were studied, analyzed and presented in Table 2 which shows all the metabolites that were tested for in each extract. Negative indicates the absence of while positive indicates the

presence of particular constituents or metabolites. All extracts tested positive for more than one phytochemical component. The MeOH and MeOH/CH<sub>2</sub>Cl<sub>2</sub> leaves extracts showed presence of all phytochemical components tested.

The phytochemical analysis of medicinal plant extracts is a preliminary step and of great importance, since it reveals the presence of bioactive constituents responsible for the therapeutic value. With this in view, and in order to establish a relationship between the anthelmintic activity of our extracts and their chemical compositions, the extracts were the subject of a quantitative phytochemical study.

Table 2. Qualitative Analysis of Phytochemicals Test.

Constituent	Leaves extracts			Roots extracts		
	H <sub>2</sub> O	MeOH	MeOH/CH <sub>2</sub> Cl <sub>2</sub>	EtOH	MeOH	MeOH/CH <sub>2</sub> Cl <sub>2</sub>
Alkaloids	-	+	+	-	-	-
Tannins	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Saponins	+	+	-	+	+	-
Triterpenes	-	+	+	-	-	-
Polyphenols	+	+	+	+	+	+
Stéroïds	-	+	+	+	-	-

(+): Presence; (-): Absence.

The phytochemicals content of the two parts of the plant are shown in Table 3. The amount of phytochemicals depend

on the plant part and the solvent used for extraction. The highest TPC, TTC and TSC was recorded for EtOH 70% roots extract ( $5.57 \pm 0.00$  mg GAE/g,  $4.07 \pm 0.2$  mg GAE/g and

$1.05 \pm 0.01$  mgEV/g respectively); While MeOH root extract had the highest TFC with  $3.56 \pm 0.20$  mgER/g.

**Table 3.** Total phenolic, flavonoid, tannins and saponins content in different *Cosmos sulphureus* extracts.

Solvents	Plant parts	TPC (mgGAE/g)	TTC (mgGAE/g)	TFC (mgER/g)	TSC (mgEV/g)
H <sub>2</sub> O	Leaves	3.61±0.00	1.90±0.20	2.03±0.00	0.93±0.01
EtOH 70%	Roots	5.57±0.00	4.07±0.20	1.33±0.20	1.05±0.01
MeOH	Leaves	3.14±0.03	2.03±0.05	2.04±0.03	0.98±0.10
	Roots	4.04±0.00	2.28±0.10	3.56±0.20	0.05±0.03
MeOH/CH <sub>2</sub> CL <sub>2</sub>	Leaves	2.60±0.04	1.93±0.00	1.04±0.06	nd
	Roots	2.97±0.10	1.89±0.02	2.18±0.00	nd

Values are means±SD; ER: rutin equivalent, GAE: Gallic acid equivalent, EV: vanillin equivalent, nd: not determined.

Active compounds produced during secondary vegetal metabolism are usually responsible for the biological properties of some plant species used throughout the globe for various purposes, including treatment of infectious diseases [33]. This study showed bioactive compounds such as phenols, tannins, flavonoids and saponins were present in leaves and roots of *C. sulphureus*. The presence of these phyto-constituents in an extract of this plant is thought to be responsible for the anthelmintic activity.

### 3.3. Worm Motility Assay

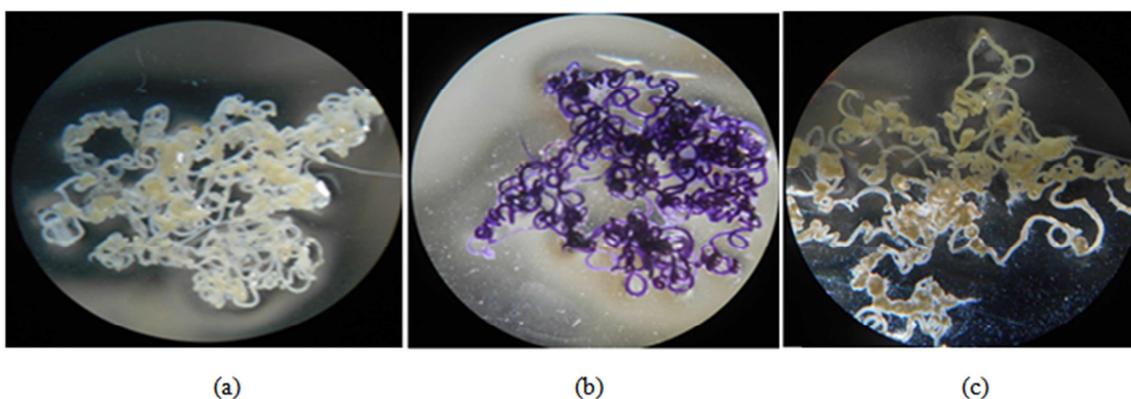
A total of 15 crude extracts prepared from the plant were used for nematocidal screening against male worms at a single concentration of 300 µg/mL 6 of these 15 extracts caused complete immobilization of the worms after 72h exposure at 37°C. Whereas in negative control (RPMI+DMSO at 0.015%), all the worms were active. Thus,

the results indicated that the inhibition in motility was faster at 6 extracts, while it was relatively slow at 9 extracts which are mostly stems extracts (Table 4). Depending on the solvent, result showed that MeOH and (MeOH) / (CH<sub>2</sub>CL<sub>2</sub>) extracts were better than other extracts.

The 6 more active extracts were selected for further studies. They were screened at six concentrations (300-50 µg/mL) on the *O. ochengi* male and female, in order to determine LC<sub>50</sub> values. The nematocidal effect of these extracts was confirmed by comparing treated worms with untreated control worms and ivermectin-treated worms. MTT is pale yellow in solution, but it is reduced by active mitochondria to yield dark blue or purple formazan within the cells when incubated with living cells. Thus, after incubation, all purple colored worms was considered as alive while all those yellow colored was considered as dead.

**Table 4.** Preliminary screening of the different extracts on *Onchocerca ochengi* males.

Plants parts	Motility (%) at 300µg/mL after 72h of incubation				
	H <sub>2</sub> O	EtO70%	MeOH	CH <sub>2</sub> CL <sub>2</sub>	MeOH/ CH <sub>2</sub> CL <sub>2</sub>
Leaves	100.00	50.00	100.00	66,67	100.00
Stems	11.11	27,78	27,78	11,11	61,11
Roots	50.00	100.00	100.00	11,11	100.00



**Figure 1.** Staining of the worms before (a) and after (b: towards alive, c: towards death) the test with the MTT.

The result suggests that the hydro-ethanolic roots extract was more active than the other extracts, even though all the extract were endowed with anthelmintic property. The type of solvent used for extraction also plays a significant role in the solubility of active compounds contained in the plant extracts.

This in turn may influence the antionchocerca activity of these extracts. The activity of these extracts against *O. ochengi* was seen to be concentration-dependent and was found to be proportional to the type of worm (figure 2).

The different LC<sub>50</sub> of the extracts are summarized in the

table 5. Data revealed that the hydro-ethanolic extract of roots showed the best anthelmintic activity with a  $LC_{50}$  of  $31.01 \pm 1.17 \mu\text{g} / \text{mL}$ . However, this extract is much more

toxic than ivermectin ( $LC_{50} = 42.78 \mu\text{g} / \text{mL}$ ). All other extracts of this plant were an activity on *O. ochengi* females, with a nematocidal ability with the male activity.

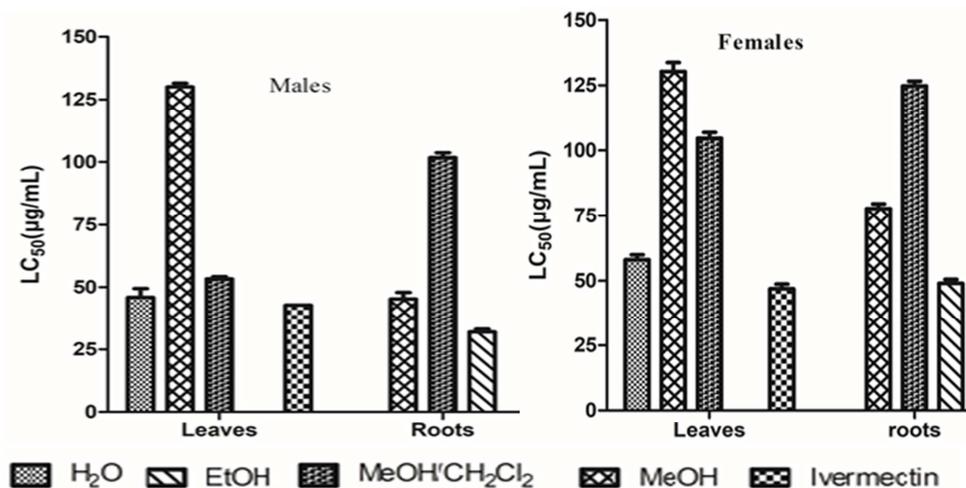


Figure 2. Comparison of  $LC_{50}$  of *Onchocerca ochengi* males (a) and females (b) exposed to different crude extracts and ivermectin.

The anti onchocerca effect of extracts is comparable with the one produced by the standard drug ivermectin. Hydro-ethanolic root extract of *C. sulphureus* exhibited significant activity and was clearly way ahead in comparison to ivermectin. This is endowed with a number of

pharmacological properties [15, 16, 17]; and its anti onchocerca potential has further added to its importance as a valuable medicinal plant. Further it can be concluded that this plant exhibited potential macrofilaricidal activity against *O. ochengi* parasite.

Table 5.  $LC_{50}$  values of different crude extracts on the mean activity of *Onchocerca ochengi* males and females.

LC <sub>50</sub> ±SEM ( $\mu\text{g} / \text{mL}$ ) after 72h							
Plant part	Leaves			Roots			Control
Solvents	H <sub>2</sub> O	MeOH	MeOH/CH <sub>2</sub> Cl <sub>2</sub>	EtOH	MeOH	MeOH/CH <sub>2</sub> Cl <sub>2</sub>	Ivermectin
Males	45.89±0.33	130.02±1.44**	53.33±0.92	31.01±1.17*	45.27±2.60	101.74±0.60	42.78±0.00
Females	58.00±1.87	130.26±3.44**	104.49±2.62**	48.91±1.59	77.79±1.81	124.73±0.49**	46.82±1.85

Values are means±SD (Standard deviation), \*\*P<0.01, a significant difference compared to the control.

Table 6. Effect of oral administration of 70% ethanol roots extract of *Cosmos sulphureus* on biochemical parameters.

Parameters	Temoim		250 mg/kg		500 mg/kg		750 mg/kg	
	Males	Females	Males	Females	Males	Females	Males	Females
ALAT (U/l)	21.53±0.67	23.61±0.01	21.67±1.75	23.42±1.53	28.08±4.70*	29.18±2.80*	33±5.11*	39.42±3.21*
ASAT (U/l)	51.37±2.09	92.49±1.74	52.64±2.01	81.04±2.66*	110.47±4.61**	106.87±5.20*	98.31±1.00**	122.22±5.04**
Protein (g/dL)	3.20±0.02	3.37±0.02	3.12±0.04	3.29±0.02	3.24±0.02	3.46±0.03	4.73±0.01	4.89±0.03
Creatinin (mg/dL)	12.93±0.03	12.00±0.00	66.00±0.04**	74.00±0.05**	25.00±0.01*	97.00±0.06**	0.00±0.00	129.00±0.9***
Glucose (mg/dL)	138.29±0.81	88.15±0.43	114.91±0.33*	130.7±0.50**	112.43±14.12*	144.30±0.76**	120.18±1.03*	85.09±0.40

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.

### 3.4. Acute and Sub-acute Toxicities Study

#### 3.4.1. Acute Toxicity

The acute toxicity studies were conducted as per the OECD guidelines 420 [32], where the limit test dose of 2000mg/kg was used. No test substance related mortality was observed at 2000 mg/kg. 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 [34]. Therefore, the results of acute toxicity in the present study show the low toxicity of a single consumption of the EtOH 70% roots extract of *C. sulphureus*.

#### 3.4.2. Subacute Toxicity

Subacute toxicity study examines toxicity caused by repeated dosing over an extended period of 28 days of oral administration in rodents. This test provides information on target organs and on the potential of the tested chemical to accumulate in the organism and then is used

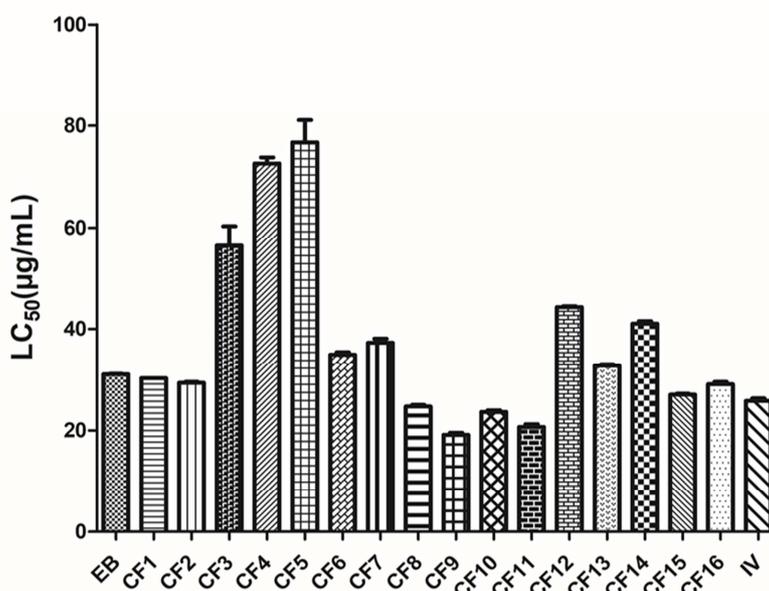
as the basis for the determination of the no observed effect level [35]. After 28 days for oral administration of hydroethanolic roots extract of *C. sulphureus* orally to mice, the biochemical parameters (except glucose) of the treated groups (250, 500, 750 mg/kg) showed a progressive decrease in values from dose 250 mg/kg to 750 mg/kg body weights. These declines were statistically different from those of the control group at  $p < 0.05$  (Table 6). The mean serum glucose level showed significant decrease ( $p < 0.05$ ) at all doses compared with the controls. In a toxic environment the blood level of AST and ALT are known to significantly increase [36]. These two classical enzymes are reliable indices of liver toxicity [37]. ALT and AST are also enzymes released as a result of liver injury, especially damage to mitochondria of liver cells. Elevation of level of these enzymes can be an indication of cellular damage, leakage and loss of functional integrity of hepatic cell membrane. Since in this study the enzymes showed no appreciable increase in the treated animals, it implied that the extract has no hepatotoxic effect.

The sub-acute toxicity of the extracts on kidney function was evaluated by using serum creatinine, as marker. In this study, significant decrease ( $P > 0.05$ ) in the level of creatinine was noted among treated male groups, against significant increase in a females treated groups, comparing 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 in male mice. This may probably be an indication that the extract did not interfere with the capacity of the kidney to excrete these metabolites. It is therefore evident that the extract at doses tested did not cause renal impairment or kidney damage.

### 3.5. Activity of Chromatographic Fraction on Male of *Onchocerca Ochengi*

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization [38]. Plant extracts usually occur as a combination of various type of bioactive compounds. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC, are used to obtain pure compounds [38]. The extraction procedure in this study resulted in obtaining concentrated preparations of bioactive substances. To achieve comprehensive knowledge of their properties, it was subjected to column and thin layer chromatographic techniques.

219 fractions of 300 mL each were collected using Hex-EtOAc and EtOAc-MeOH as eluent. The fractions were pooled on the basis of their TLC profiles to give a total of 16 combined fractions (CF1-16). These 16 fractions were tested on parasite males and the result (Figure 3) demonstrated anthelmintic susceptibility in all fractions. However, the highest activity was observed with fractions CF8 (Hex/AcOEt 70%), CF9 (Hex/AcOEt 80%), CF10 (Hex/AcOEt 90%) and CF11 (AcOEt 100%). They exhibited lower  $LC_{50}$  values (24.66; 19.10; 23.61 and 20.65  $\mu\text{g}/\text{mL}$  respectively) than that of the crude extract and the ivermectin (31.01 and 25.77  $\mu\text{g}/\text{mL}$  respectively). While, lower activity was observed with fractions CF3 (Hex/AcOEt 20%), CF4 (Hex/AcOEt 30%), CF5 (Hex/AcOEt 40%), CF12 (AcOEt/MeOH 10%) and CF14 (AcOEt/MeOH 30%).



**Figure 3.** Nematocidal activity of fractions derived from *Cosmos sulphureus* roots expressed in lethal concentration 50 (50% mortality) on *Onchocerca ochengi* male; EB: crude extract; IV: ivermectin.

Once an extract is separated into several fractions and the parent extract and fractions are tested in an assay several

outcomes are possible. One outcome is that all activity may be lost in the daughter fractions, in which case the separation method is deemed unsuitable. Loss of biological activity may be due to irreversible binding to the separation media, or to instability of the active compound. A second outcome would be for all or most daughter fractions to have some low amount of activity. This too is undesirable and simply indicates that the separation mode is not suitable. The third and desired outcome is that one or several daughter fractions contain substantial bioactivity, and that the mass of active fractions has been reduced from the parent with a corresponding increase in potency [39]. In the present study, the third outcome is clearly established in fractions 8, 9, 10 and 11. Most plants contain several compounds with anthelmintic properties: Ellagic and gentisic acids, were isolated from the axlewood tree, *Anogeissus leiocarpus* [40]; Gallic acid analogues were isolated from the fruits of *Acacia nilotica* from the northern areas of Cameroon [41]. (+)-catechin-3-O-gallate, and four related proanthocyanidins: (+)-epicatechin-3-O-gallate, (+)-gallocatechin, (-)-epigallocatechin and (-)-epigallocatechin-3-O-gallate were all isolated and evaluated against *C. elegans* and *O. ochengi*. 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). Not all action mechanisms work on specific targets, and some sites may be affected due to other mechanisms. The degree of concordance between traditional use of *C. sulphureus* and the observed anthelmintic properties of this study suggest that there may be some truth to this remedy.

#### 4. Conclusion

In the *in vitro* anthelmintic activity evaluation of *C. sulphureus* against *O. ochengi*, it can be deduced that the hydro-ethanolic crude extract of roots showed highly consist anthelmintic properties more than all other extracts. Moreover, the strong anthelmintic activities exhibited by some of its fractions after separation is a proof that the plant, which was traditionally used for treatment worm infections can be a potential source of natural anthelmintics. However, further research is needed toward further isolation and identification of active principles present in the fractions, establish their mechanisms of action and perform *in vivo* anthelmintic assay.

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