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Phytochemical Analysis and Anthelmintic Activity of Ethanolic Extracts of Gmelina arborea on the Bovine Parasite *Onchocerca ochengi*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was aimed at analyzing the phytochemical constituents, evaluated the *in vitro* filaricidal activities of the ethanolic extracts of *G. arborea* on *Onchocerca ochengi* and carried out the acute and sub-acute toxicity on rats.
Place and Duration of Study: The work took place at the Laboratory of Applied Zoology of the Faculty of Science of the University of Ngaoundere between June 2020 and April 2021.
Methodology: Quantitative determination of total phenols, total flavonoids and tannins were performed by using standard titration curve of gallic acid and rutin. The adult male worms of *O. ochengi* were incubated in Roosevelt Park Memorial Institute (RPMI-1640) medium supplemented with antibiotics containing different concentrations of the plant extracts and ivermectin (positive control). The mortality of worms was evaluated using the MTT/formazan colourimetry test after 24 h and 48 h. Acute and sub-acute toxicity tests of *G. arborea* were evaluated on rats according to OECD 425 and 407 respectively.
Results: The quantitative phyto-constituents screening revealed that total phenols (873.33 ± 3.33 mg GAE/g) were the most quantified compound followed by tannins (229.33 ± 0.33 mg GAE/g) and

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total flavonoids (116 ± 1.01 mg RE/g) in the roots of the plant. 0.22 ± 0.02 mg/mL, 0.17 ± 0.00 mg/mL, 0.16 ± 0.01 and 0.11 ± 0.01 mg/mL were respectively the LC_{50} of hydroethanolic extracts of the leaves, barks, roots and ivermectin at 48 h. The acute and sub-acute toxicity test showed no toxicity in rats.

Conclusion: Looking at the result, the extracts of this plant may be used in novel drug discovery to fight against onchocerciasis.

Keywords: Onchocerca ochengi; Gmelina arborea; phytochemical constituents; toxicity; anthelmintic.

1. INTRODUCTION

Human onchocerciasis or river blindness is a parasitic disease caused by the filarial nematode *Onchocerca volvulus* [1]. The parasite is transmitted from host to host by the blood-feeding blackfly *Simulium* [2]. About 21 million persons are infected; 240 million are at risk in the world and close to 99 % of all the patients live in Tropical Africa [3]. Pathologically, the disease is associated with extensive and disfiguring skin changes, musculoskeletal complaints, weight loss, and changes in the immune system [4]. Onchocerciasis forces the affected population to abandon endemic areas which usually have high agricultural potential [5], leading to economic loss and slowdown of the country development over the years [6]. Several approaches have been attempted to control onchocerciasis in Human. Actually the key recommended chemotherapeutic molecule against onchocerciasis agent currently in use is ivermectin (Mectizan®, Merck) [7]. It is rather unfortunate, this drug is only microfilaricide, and also kills *Loa loa* microfilariae in blood, a situation that often leads to encephalopathy in patients with high *L. loa* microfilariae load [8]. This drug is also used as long-term treatment until the killing of all adult worms, which can live for up to 15 years. This may lead to patients' noncompliance. The continuous use of ivermectin for onchocerciasis control has lead to *O. volvulus* resistance to ivermectin in some communities in Ghana and Sudan [9]. Therefore, the search for new, safe and effective onchocerciasis drugs is imperative. Herbal medications play a central role in the cure of several diseases particularly in developing countries [10]. 80% of the population in Africa still do not have access to, or cannot afford to pay for synthetic anthelmintics. They depend on traditional herbal medicine for primary health care needs [11]. Filaricidal activities have been detected in some plant families which were investigated for anti-*Onchocerca* activity [12]. These plants were assessed as potential sources of drug to give alternatives to the limitations of current drugs by making phytomedicines which have anti-parasitic activities [13].

Traditional healers in Cameroon use *Gmelina arborea* for the treatment of human diseases in the northern part of country. The root and bark of *G. arborea* are been used in traditional medicine against hallucinations, piles, abdominal pains, burning sensation, fever and urinary discharges [14]. The pharmacological activity studies of this plant revealed that it possesses antioxydants activity [15], antidiurectic [16], anthelminthic against some parasite like *Pheretima posthuma* and *Ascardia galii* [17], antidiabetic [18] and antimicrobial activities against some fungi and bacteria [19]. However, the anthelmintic activity of this plant has not yet been evaluated on filarial worms. *Onchocerca ochengi* considered as an appropriate model to study anthelmintic activities found exclusively in cow, is the closest relative to *O. volvulus*. In this study, we investigated the claimed phytochemical composition, evaluated *in vitro* filaricidal activities of *G. arborea* against the parasite *O. ochengi* and evaluated its toxicity.

2. MATERIAL AND METHODS

2.1 Sample Collection and Identification

The leaves, barks and roots of *G. arborea* (Verbenaceae) were collected in Ngaoundere, Vina Sub-Division; Adamawa Region of Cameroon and identified by Pr. Tchobsala, at the Department of Biological Sciences of the University of Ngaoundere, Cameroon. Voucher specimens were being registered under the No 18581 SRF/Cam at the National Herbarium in Yaounde (Cameroon). 1 kg of the leaves, bark and roots of the plant (5 to 6 young and olds) were sampled and were dried at room temperature, weighed, ground finely, sieved on a 0.5 mm mesh screen. The resulting powder was stored until the extract preparation. All other chemicals were obtained from Sigma (Deisenhofen, Germany).

2.2 Preparation of Plant Extracts

Fifty grams of the powdered material were extracted with 500 mL in ethanol-distilled water (70:30) % for 48 h at room temperature, centrifuged (3500g, 10 min) and filtered over filter papers (5891blackribbon, ashless, Schleicher Company). The clear filtrate was concentrated by a rotary evaporator at 40 ^{o}C (BUCHI Rotavapor R-200, Switzerland). The resulting powder was stored at 4 ^{o}C (fridge BOMANN) for future investigation. The dried plants extracts were diluted with 100 % dimethylsulphoxide (DMSO) and distilled water to final concentration of 100 mg/mL. The solution was mixed thoroughly and stored at 4 ^{o}C (fridge BOMANN) for anthelmintic activity determination against *O. ochengi* using the protocol of Cho-Ngwa et al. [20].

2.3 Quantitative Phytochemical Analysis

The method of Folin-Ciocalteu (FC reagent) was used to evaluate total phenols content. Briefly, 50 µL of the ethanolic extracts of *G. arborea* were mixed with 200 µL of 35 % (w/v) Na_2CO_3 and 250 µL of 1/10 (v/v) FC reagent. The mixture was agitated and incubated in darkness at 40 °C for 30 min and the absorbance was read at 765 nm using a spectrophotometer (UV-biowave Cambridge, England). The results were expressed in mg equivalent of gallic acid per grams of dry materials (mg of GAE/g) [21]. Total phenols quantity was determined by calculation from the standard curve of gallic acid titration. All the experiments were carried out in triplicate.

The method described by Dehpour et al. [22] was used to determine flavonoids content. Briefly, 500 µL of the ethanolic extracts of *G. arborea* were mixed with 1500 µL of 95 % methanol, 100 µL of 10 % (m/v) $AlCl_3$, 100 µL of 1 M acetate and 2.8 mL distiller water. The mixture was agitated and incubated in darkness for 30 min and the absorbance was read at 415 nm using a spectrophotometer (UV-biowave Cambridge, England). A standard titration curve was made using rutin titration curve was set at the absorbance of 430 nm and the flavonoids amount was expressed as mg equivalent of rutin per grams of dry materials (mg of RE/g). All the experiments were carried out in triplicate.

The tannins content was carried out using the method described by Wolfe et al. [23]. Briefly, 200 µL of the ethanolic extracts of *G. arborea* were mixed with 35% (w/v) Na_2CO_3 and 100 µL of Folin-Ciocalteu (FC) reagent. The solution was vortexed for one minute, incubated for five minutes and the absorbance at 640 nm was then measured. The results were expressed in mg equivalent of gallic acid per gram of dry materials (mg of GAE/g). All the experiments were carried out in triplicate.

2.4 Tests on *Onchocerca ochengi*

2.4.1 Isolation of *Onchocerca ochengi*

The isolation of *O. ochengi* adult worms was done using the method described by Ndjonka et al. [24]. Briefly, fresh pieces of infected cattle umbilical skin with palpable nodules bought from the slaughterhouse at Ngaoundere were brought to the laboratory washed, drained and sterilized with 70 % ethanol. Nodules of *O. ochengi* were removed from the skin by dissection. After dissection of the nodules, adult male *O. ochengi* worms were isolated and washed in sterile phosphate-buffered saline (PBS). Worms were subsequently transferred to RPMI-1640 (Roosevelt Memorial Park Medium -1640, SIGMA, USA)

2.4.2 *In vitro* anthelmintic assay of plant extracts and ivermectin on *onchocerca ochengi* adults

Anthelmintic tests of ethanolic extracts of *G. arborea* were performed following the protocol of Cho-Ngwa et al. [25]. Briefly, solutions of each part of the plant extracts and ivermectin were prepared with RPMI-1640 supplemented with penicillin and streptomycin (100 U/100 µg/mL) at 6 different concentrations (0.1, 0.2, 0.4, 0.5; 0.75 and 1 mg/ml) and 0.5 mL of each of these solutions was deposited in the wells of culture plates (24-well plates). Then, 6 individuals were placed in each well. (6 worms per well). Assays were done in independents triplicate determinations. Samples were incubated at 37 ^{o}C in a CO2 incubator and the mortality was determined after 24 h and 48 h by the MTT/ formazan assay.

2.4.3 Biochemical determination of worm viability and lc_{50} determination

To determine mortality of the worms, the worms were removed from their wells, washed with PBS and subjected to the MTT test (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium

bromide). The MTT is a pale yellow compound which is reduced to a dark blue or purple product, formazan by the living cells of the worms. Single intact worms were placed in each well of a 26-well plate containing 500 µL of a solution consisting 450 µL MTT (0.5 mg/mL) and 50 µL of RPMI, then incubated at 37°C and observed after 30 min under a binocular microscope. Thus, after incubation, the worms that were alive were colored in purple because the MTT was reduced to formazan. Dead worms do not reduce MTT to formazan but simply take on the yellow color of MTT. All MTT assays were done in the dark since the MTT reagent is sensitive to light [26]. The lethal concentration 50 (LC_{50}) was calculated using Log-probit method with SPSS version 16.0 Software.

2.5 Acute and Sub-acute Toxicity Tests of *G. arborea* in Wistar Rats

2.5.1 Acute toxicity test of *g. arborea* in wistar rats

Wistar rats weighing between 90 ± 10 g were purchased from LANAVET (National Veterinary Laboratory) in the North-Region (Cameroon). Animals were housed and maintained under ambient temperatures (25 ± 3°C) with relative humidity of 55 ± 1 °C where they were acclimatized to standard laboratory conditions for 5 days. Experiments on the animals were authorized by the regional delegate of livestock; fisheries and animal industries (N° 075/16/L/RA/DREPIA). Acute oral toxicity study was carried out according to the OECD 423 Guidelines for chemicals assays at the single limit dose of 2000 mg/kg [27]. Prior to the administration of plant extract, animals were withheld from food overnight but not from water. The animals were weighed and divided into two groups (01 treated group and 01 control group) of three individuals each. Then, 2000 mg/kg dose of the extract of ethanolic roots of *G. arborea* was orally given to the treated groups in a single dose/exposure, while the untreated group received only distilled water. After administration of the extracts, food was withheld for 2 h, effect on grooming, hyper activity, sedation respiratory arrest, convulsions, motor activity and mortality were observed every 30 min for 4 h on the first day and once daily for 14 days.

2.5.2 Sub-acute oral toxicity

The sub-acute oral toxicity of the roots of *G. arborea* ethanolic extracts was assessed according to the OECD Guideline No. 407 [28]. This study was evaluated on 24 Wistar rats of both sexes weighing between 77 and 108 g. Animals were randomly separated into three test groups and a control group (n = 6; three males + three females). Rats were kept fasting for 12 h providing only water, which the extracts were administrated at the dose of 1 mL/100g of body weight to the groups tests and control group. The extract was dissolved in distilled water and orally administered to the rats daily for 28 days at single doses of 250, 500, and 1000 mg/kg. The control group received only distilled water. Their weights were taken before administration of the extract and then every 7 days. Weight changes were also recorded weekly during the observation period. At the end of the toxicity studies, the rats were fasted overnight, sacrificed to perform haematological, biochemical, and histological analyses.

2.6 Blood Analyses

Blood samples were drawn by cardiac puncture for haematological and biochemical investigations. They were transferred to heparinized tubes containing ethylenediaminetetraacetic acid (EDTA) for the evaluation of haematological parameters and to non-heparinised tubes for biochemical examinations. The tubes were centrifuged at 3000× g at 4 ^{o}C for 10 min to separate the sera, which were then stored at −20 ^{o}C until subsequent analyses [29].

2.6.1 Haematological assay

The blood samples in the heparinized tubes were used for haematological analyses including white blood cell count (WBC), lymphocytes, monocytes, granulocytes, red blood cell count (RBC), hemoglobin (Hb), hematocrit, platelet count, mean corpuscular volume (MCV) and Mean hemoglobin concentration (MHC). The measurements were made with a total autoanalyser/automated biochemistry analyser (Mindray Automatic Analyzerfi, BA-884) [30].

2.6.2 Biochemical analysis

Sera were collected to measure aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), albumin (ALB) for liver function and creatinine (Crea) and Urea for renal function using a LR SGMitalia kit according to the manufacturer's instructions Zaida et al [31].

2.6.3 Histopathological examination

After blood collection on day 29, all animals were euthanized for gross pathological examinations of all major internal organs. The hearts, livers, lungs, kidneys, and spleens were excised and weighed. The relative organ weights were calculated as follows:

ROW= (organ weight (g) / body weight of experimental rat on the day of sacrifice (g)) × 100.

The vital organs were maintained in 10% (v/v) formalin. The organs of interest were trimmed, embedded in paraffin, and sectioned into slices of 5 µm thickness. The tissue sections were stained with haematoxylin and eosin and inspected under a light microscope for histopathological changes. The microscopic features of the organs of treated groups were compared with those of the control group [32].

2.7 Statistics

Results are presented as mean values ± standard error of the mean (SE) using GraphPad prism version 6.01 Software. Error bars in bar graphs represent SEM. The lethal concentration 50 was calculated using Log-probit method with SPSS version 16.0 Software. LC_{50}, means for biochemical and haematological parameters and body and organ weights, comparison was done using analysis of variances (two ways - ANOVA) followed by the multiple tests of comparison of Bonferroni. 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 $P \leq 0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The results in Table 1 shows that total phenols are more abundant in the roots ($P < 0.001$) compared to the bark and the leaves of the plant. In the bark and the leaves, total phenols content are the same ($P > 0.05$). Flavonoids are abundant in the leaves and in the bark ($P < 0.001$) compared to the roots. Tannins are abundant in the roots ($P < 0.001$) compared to leaves and the barks.

3.2 Activities of Plant Extracts and Ivermectin on *Onchorcerca ochengi* Adult Worms

The ethanolic extracts of leaves, barks and roots from *G. arborea* showed an anthelmintic activity on *O. ochengi* adult male worms at 24 and 48 h of incubation at 37°C. Mortality rates of *O. ochengi* at 24 h and 48 h after incubation are presented in Fig. 1. These results revealed that the extracts of leaves, barks and roots recorded a case of mortality at the concentration of 0.1 mg/mL for 24 h and 48 h of incubation. The 100 % of mortality is observed after 48 h of incubation at the concentration of 0.75 mg/mL for the extracts of leaves and roots and at the concentration of 1 mg/mL for the barks (Fig. 1a,b,c). For ivermectin 100 % mortality was observed after 24 h and 48 h at the concentrations of 0.75 and 0.5 mg/mL (Fig. 1d).

Table 2 showed that at 24 h, there is neither a significant difference between the extracts of the roots of the plant nor the reference drug ($P > 0.05$) but; a significant difference was observed between the leaves and the reference drug ($P < 0.001$) and between the barks and ivermectin ($P < 0.01$). At 48 h; there is neither a significant difference between the extracts of the roots and the reference drug ($P > 0.05$) with however a significant difference between the leaves and the reference drug ($P < 0.001$) and between the barks and ivermectin ($P < 0.05$). This study revealed that the roots extracts have the best anthelmintic activity with a LC_{50} of 0.17 ± 0.01 mg/mL. In comparison to the LC_{50} of Ivermectin, no significant difference was observed between the two ($P > 0.05$).

Table 1. Quantity (mg) of phytochemical compounds (total phenols, flavonoids and tannins)

	Total phenols (mgGAE/g)	Flavonoids (mgRE/g)	Tannins (mgGAE/g)
Leaves	496.66 ± 13.33^a	333.33 ± 1.85^a	56.67 ± 0.33^a
Barks	496.66 ± 8.81^a	276.66 ± 6.66^a	16.33 ± 3.17^a
Roots	873.33 ± 3.33^b	116 ± 1.01^b	229.33 ± 0.33^b

Values are means ± SE; means in each column followed by same letters are not significant; a: No significant difference (P > 0.05); b = significant difference (P < 0.001), GAE: Gallic acid equivalent, RE: rutin equivalent

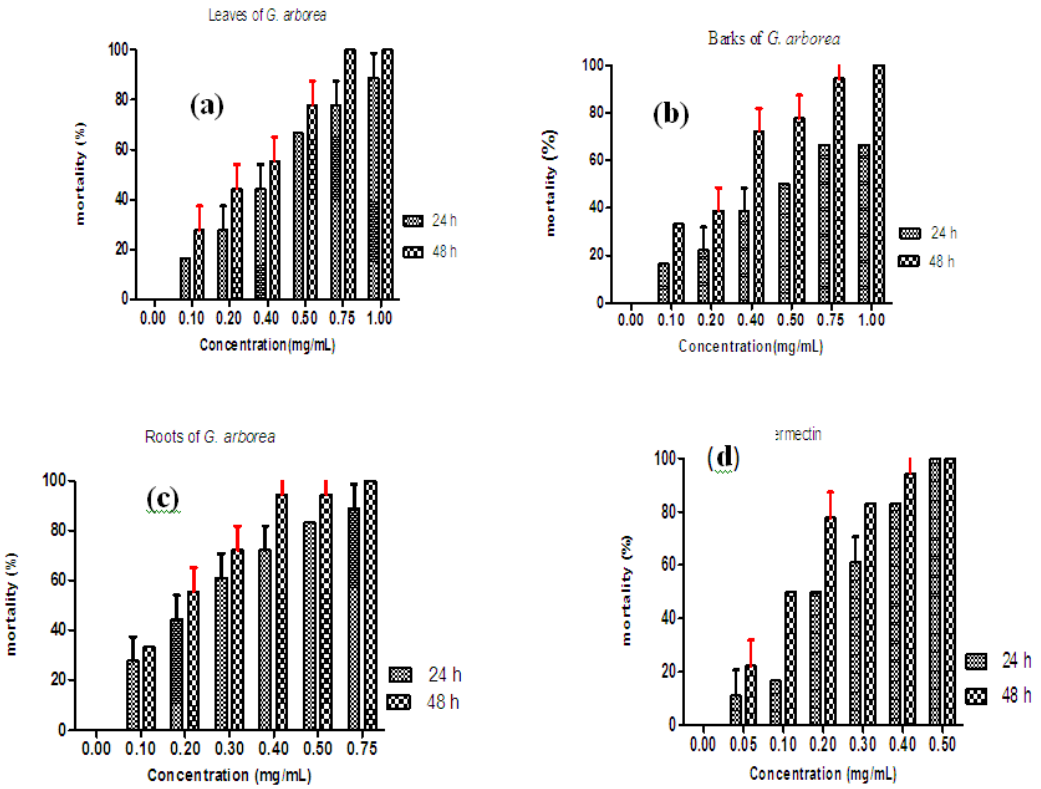

Fig. 1. Mortality percentage of *O. ochengi* exposure to increasing concentrations (0-1 mg/mL). (a) ethanolic extract of *G. arborea* leaves, (b) ethanolic extract of G. *arborea* bark, (c) ethanolic extract of *G. arborea* roots, (d) ivermectin

Table 2. LC_{50} of the ethanolic extracts of *Gmelina arborea* against *Onchocerca ochengi* compared to ivermectin after 24 and 48 h exposure

	24 h	48 h
	CL_{50} (mg/mL)	CL_{50} (mg/mL)
Leaves	0.33 ± 0.02^{b}	0.22 ± 0.02^{b}
Barks	0.26 ± 0.12^{c}	0.17 ± 0.00^{d}
Roots	0.19 ± 0.01^{a}	0.16 ± 0.01^{a}
Ivermectin	$0.17 \pm 0,01^{a}$	$0,11 \pm 0.01^{a}$

Values are means ± SE; means in each column followed by same letters are not significant; a: No significant difference (P > 0.05); b = significant difference (P <0 .001); c = significant difference (P < 0.01); d = significant difference (P < 0.05)

3.3 Toxicity Test

3.3.1 Acute toxicity test

In the acute toxicity test, the oral administration of the roots of *G. arborea* ethanolic extracts on the rats showed that there were no changes in the behavioral pattern such as grooming, hyper activity, sedation respiratory arrest, convulsions, and motor activity compared to rats in the control group. No mortality was recorded for the 14 days' observation period. So, the DL_{50} value of the tested extract was up to 2000 mg/kg, and this puts the extracts in category 5 toxicity class according to the Globally Harmonised System (GHS) of classifying substances (OCDE 423) which are regarded as relatively safe substances.

3.3.2 Subacute toxicity

3.3.2.1 Effects of sub-acute of the ethanolic extracts of the roots of gmelina arborea on rat body weights

Table 3 shows the effect of sub-acute toxicity of the ethanolic extracts of the roots of *G. arborea* on the weight of male and female rats. As shown on Table 3, male and female rats in both the control and treated groups showed progressive increase in body weight over the course of the period of treatment. There was no significant difference in weights of *G. arborea* treated rats compared to the control group.

3.3.2.2 Effect of oral administration of Gmelina arborea roots extract on Haematological Parameters

The effect of *G. arborea* roots extract on the haematological parameters of the experimental and control groups of rats is shown in Table 4. In this study, there were no significant differences in White Blood Cells, Lymphocytes, monocytes, Granulocytes, Red blood cells, Haemoglobin, Hematocrit, mean corpuscular volume and Mean hemoglobin concentration during the 28 days' treatment period ($P > 0.05$) at all the tested doses for both sexes (Table 4). However, there was a significant increase in the values of Platelets in the experimental group which received the doses of 500 mg/kg and 1000 mg/kg as compared with control groups for males ($P < 0.001$). The platelets increased in a dose-dependent manner during the treatment period. The values of platelets passed from 561 ± 53.72 (× 10/L) in the control group to 701.33 ± 36.09 (× 10/L) and to 692.33 ± 16.42 (× 10/L) in the males treated group respectively with 500 mg/kg and 1000 mg/kg, representing 25.01 % and 23.4 % respectively.

3.3.2.3 Effect of oral administration of gmelina arborea roots extract on biochemical parameters of rats after 28 days of treatment

In the female rats, there was no significant difference found between the control and the different doses of the extract for ASAT, ALAT and Urea (Table 5). Regarding creatinine and albumin, no significant difference was observed in the treated groups compared with the control group. In the male rats, regarding ASAT, ALAT and Urea a statistical difference was observed between the treated groups at different doses of the ethanolic extracts of *G. arborea* compared with the control group (Table 5). The three doses of the extract induced significant decrease in the ASAT, ALAT and Urea values in the males treated groups. With the ASAT, there was a significant reduction of around 61.34 %, 89.93 % and 88.47 % respectively at doses of 250; 500; 1000 mg/kg. For Urea, there was a significant reduction in the order of 67.77 %; 32 %; 14.44 % respectively at doses of 250; 500 and 1000 mg/kg.

Table 3. Body weigth (g) of male and female rats in sub-acute toxicity of the roots of *G. arborea*

Treatment	Sex	Intial	7 days	14 days	21 days	27 days
Distilled water	M	96.00 ± 3.51	109.67 ± 2.33	129.67 ± 0.88	145.33 ±2.18	154.33 ± 4.67
	F	85.67 ± 4.49	99.33 ± 4.05	119.67 ± 2.03	131.00 ± 1.20	136.67 ± 1.20
250 mg/kg	M	108.33 ± 3.18	121.33 ± 0.88	135.33 ± 2.67	161.00 ± 2.52	165.67 ± 2.18
	F	96.00 ± 1.00	108.67 ± 2.40	128.33 ± 2.60	134.67 ± 2.60	146.33 ± 2.67
500 mg/kg	M	97.00 ± 3.21	118.00± 13.05	132.67 ± 16.42	160.33± 22.28	167.67 ± 22.82
	F	85.67 ± 4.67	96.00 ± 5.29	110.00 ± 3.51	122.33 ± 2.84	124.33 ± 0.67
1000 mg/kg	M	83.00 ± 3.05	124.00 ±3.78	149.67 ± 7.96	171.00 ± 9.54	173.33 ± 7.75
	F	82.00 ± 3.51	96.33 ± 2.72	116.33 ± 2.91	125.00 ± 2.09	127.33 ± 1.86

Values are means ± SE; with each batch comprising 6 animals. M : male and F : female. no statistically significant differences were found between the control and the different doses of the extract at P > 0.05

Table 4. Effect of the ethanolic extract of *Gmelina arborea* on haematological parameters values in the repeated dose toxicity study

Parameters	Sex	Treatment group			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
WBC Count	M	8.56 ± 2.47	5.36 ± 1.68	8.90 ± 1.17	6.50 ± 0.21
(× 10^9/L)	F	7.93 ± 1.52	5.93 ± 0.54	8.37 ±1.19	8.13 ± 0.77
Lymphocytes (%)	M	83.2 ± 1.45	85.66 ± 1.51	76.60 ± 3.27	74.36 ± 3.07
	F	81.133 ± 4.15	87.60 ± 3.41	80.63 ± 4.85	81.70 ± 4.02
Monocytes (%)	M	10.16 ± 2.30	8.50 ± 3.25	8.50 ± 0.94	8.93 ± 1.71
	F	6.63 ± 1.18	5.20 ±1.57	9.93 ± 2.79	7.10 ±1.00
Granulocytes (%)	M	9.96 ± 2.54	15.83 ± 7.69	14.90 ± 3.00	16.70 ± 3.46
	F	11.9 ± 3.54	7.20 ±1.88	9.43 ± 2.15	11.20 ± 3.05
Red Blood Cells	M	6.98 ±0.33	5.27 ± 2.34	7.28 ± 0.39	7.40 ± 0.17
(× 10/L)	F	6.28 ± 0.10	6.96 ± 0.14	5.85 ±1.37	7.09 ± 0.61
Haemoglobin	M	13.43 ± 0.54	12.16 ± 2.03	13.96 ± 0.93	14.23 ± 0.27
(g /dL)	F	14.3 ± 0.23	14.00 ± 0.26	13.03±0.77	14.13 ±1.37
Hematocrit,(%)	M	43.00 ± 2.36	43.40 ±1.40	41.36 ± 2.26	41.10 ± 1.05
	F	43.67 ± 1.19	41.77 ± 0.88	34.83 ±7.60	41.27 ± 3.88
Platelets (× 10/L)	M	561 ± 53.72	554.33± 66.33	701.33 ± 36.09 [b]	692.33 ± 16.42 [b]
	F	644.67 ± 97.51	605.33 ± 89.91	611.76 ± 36.09	598.00 ± 47.37
MCV (fL)	M	61.53 ± 1.32	56.60 ± 0.55	56.76 ± 0.35	55.53 ± 0.77
	F	60.4 ± 0.74	60.00 ± 0.15	60.17 ±1.49	58.07 ± 0.63
MHC (pg)	M	19.3 ± 0.50	18.46 ± 0.21	19.13 ± 0.49	19.26 ± 0.14
	F	19.8 ± 0.8	20.13 ±0.45	24.93 ± 8.41	19.86 ± 0.33

Values are means ± SE; with each batch comprising 6 animals. M: male and F: female ; b = significant differences (P < 0.001) were found between the control and the different doses of the extract for these parameters

Table 5. Effect of the ethanolic extract of *Gmelina arborea* on biochemical parameters values in the repeated dose toxicity study

Parameters	Sex	Treatment group			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
ASAT (IU/L)	M	102.67 ± 2.41	39.67 ± 2.18 [b]	10.33 ± 1.85 [b]	11.83 ± 1.96 [b]
	F	87.67 ± 3.93	88.66 ± 4.66	95.66 ± 2.03	96.66 ± 2.03
ALAT (IU/L)	M	100.33 ± 1.45	25.00 ± 2.64 [b]	10.33 ± 2.02 [b]	11.33 ± 1.76 [b]
	F	78.67 ± 4.67	71.66 ± 4.41	78.66 ± 8.66	91.01 ± 5.86
Urea (mg/L)	M	78.5 ± 6.75	25.30 ± 1.26	53.23 ± 0.61 [b]	67.16 ± 2.68 [b]
	F	22.57 ± 3.82	21.97 ± 3.29	36.07 ± 0.48	30.40 ± 3.06
Creatinine	M	2.0 ± 0.20	0.53 ± 0.03	1.63 ± 0.09	1.27 ± 0.53
(mg/L)	F	0.5 ± 0.10	0.70 ± 0.05	0.87 ± 0.32	0.33 ± 0.23
Albumin (g/L)	M	45.03 ± 2.58	44.30 ± 1.20	42.10 ± 1.49	41.66 ± 0.99
	F	39.7 ± 2.56	44.53 ± 2.12	35.30 ± 2.69	40.73 ± 2.37

Values are means ± SE; with each batch comprising 6 animals. M: male and F: female ; b = significant differences (P < 0.001) were found between the control and the different doses of the extract for these parameters

3.3.2.4 Relative organ weight

Relative organ weight values of all animals are presented in Table 6. In males, no statistical significant difference (P > 0.05) was observed between the relative weight of the liver, heart, lungs, kidneys and spleen of the different batches that received the different doses of the extract compared to the control group. In females, no statistical significant difference (P > 0.05) was found between the relative weight of the heart, lungs, kidneys and spleen of the different groups that received the different doses of the extract and the control. However, for the liver of rats based on the different doses, a significant increase in relative weight was observed (P < 0.01) compared to the control group.

Table 6. Relative organ weights of male and female rats after 28 days of oral treatment

Parameters	Sex	Treatment group			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
Heart	M	0.391 ± 0.022	0.385 ± 0.005	0.364 ± 0.021	0.315 ± 0.164
	F	0.449 ± 0.018	0.521 ± 0.037	0.427 ± 0.026	0.436 ± 0.004
Lungs	M	1.064 ± 0.047	1.075 ± 0.063	1.064 ± 0.036	1.019 ± 0.050
	F	1.121 ± 0.029	1.187 ± 0.069	1.243 ± 0.061	1.243 ± 0.004
Kidney	M	1.022 ± 0.052	0.971 ± 0.041	1.030 ± 0.082	1.039 ± 0.034
	F	1.241 ± 0.013	1.251 ± 0.017	1.247 ± 0.012	1.251 ± 0.031
Liver	M	5.508 ± 0.390	4.881 ± 0.390	5.196 ± 0.391	4.862 ± 0.131
	F	5.010 ± 0.094	5.677 ± 0.138	5.731 ± 0.170 [b]	5.764 ± 0.100 [b]
Spleen	M	0.560 ± 0.048	0.292 ± 0.021	0.363 ± 0.037	0.249 ± 0.006
	F	0.381 ± 0.037	0.349 ± 0.009	0.382 ± 0.022	0.348 ± 0.013

Values are means ± SE; with each batch comprising 6 animals. M : male and F : female. no statistically significant differences were found between the control and the different doses of the extract at P > 0.05

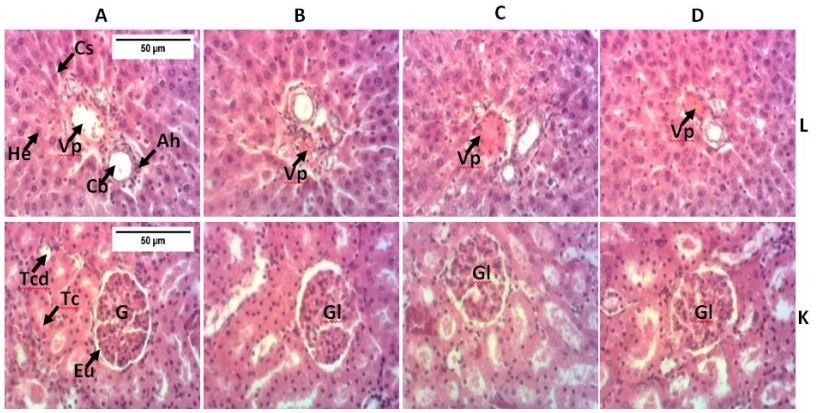

Fig. 2. Liver (L) and kidney (K) microphotographic sections (X200) of male rats showing effects of oral administration of the *Gmelina arborea* extract for over 28 days; cells were stained with hematoxylin and eosin (H&E, 200×)

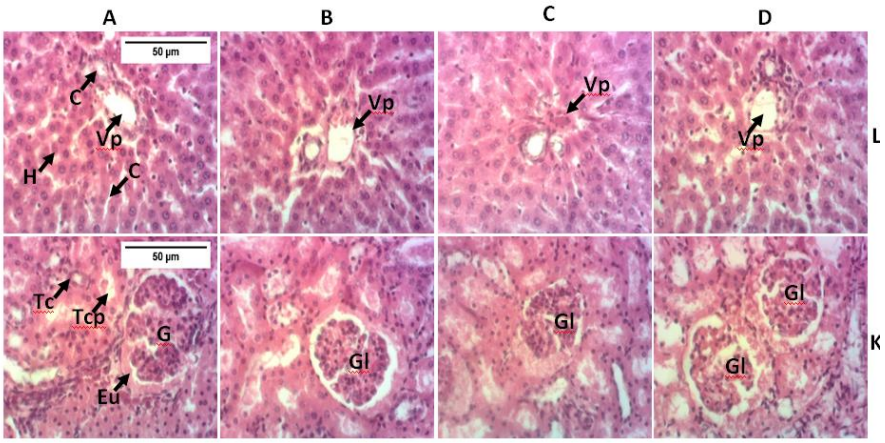

Fig. 3. Liver (L) and kidney (K) microphotographic sections (X200) of female rats showing effects of oral administration of the *Gmelina arborea* extract for over 28 days; cells were stained with hematoxylin and eosin (H&E, 200×). A = control group; B = 250 mg/kg; C = 500 mg/kg; D = 1000 mg/kg. Indicators; (L) = Liver, K = kidney; Liver; Vp = hepatic portal vein; He = hepatocytes; Cs = sinusoidal Capillary; Cb = bile canaliculi; Kidney; Gl = glomerulus; Eu = urinary space; Tcd = distal convoluted tubule; Tcp = proximal convoluted tubule

3.3.2.5 Histopathology of the liver and kidneys

Histopathological examination was performed on the kidneys and liver of the male and female rats to detect morphological damages on them. The histological sections performed are presented in Fig. 2 and 3 for the male and female rats respectively. Observation of these cuts did not reveal any abnormalities like lesions or other pathological changes on the liver and kidneys at the doses of 250, 500, 1000 mg/kg body weight/day in both sexes. The treated group of rats presented intact hepatocytes, portal veins, glomeruli, and tubules like the control group.

4. DISCUSSION

This work aimed at analyzing the phytochemical constituents, evaluated the *in vitro* filaricidal activities of the ethanolic extracts of *G. arborea* on *Onchocerca ochengi* an experimental model of *Onchocerca volvulus* and to carry out the acute and sub-acute toxicity on rats. The determination of phytochemical constituents of different parts of the plant has revealed that total phenols are abundant in the roots followed by bark and the leaves. Flavonoids are abundant in the leaves and in the bark. Tannins are abundant in the roots followed by leaves and the barks. The high quantity of these secondary metabolites may be due to the solvent used because, it is known that by adding water to pure ethanol up to 30 % to prepare ethanol 70 % the polarity of solvent was increased [33]. To Add, it was discovered that ethanol easily penetrates the cellular membrane to extract the intracellular ingredients from the plant material [34]. A study carried out by Offor [35] showed that the leaves of *G. arborea* have a lower concentration of total phenols (0.32 ± 0.01 mg/100g) and flavonoids (0.06 ± 0.01 mg/100g). These differences observed between the values of these bioactive constituents and those of other studies depends on factors such as climate, soil physico-chimics constituents, the nature of the solvent used, part of the plant and geographical location of plants [36]. In a study conducted by Ndjonka et al. [37] and Muda et al. [38] show that the activity of a plant extract depends on the availability of secondary metabolites like tannins; saponins; polyphenols and flavonoids might be responsible for the anthelmintic activity. It is the presence of total phenols, flavonoids and tannins in high concentration which are responsible for anthelmintic activities of the ethanolic extracts of *G. arborea*.

The results for the *in vitro* anthelmintic activity of the ethanolic extracts of *G. arborea* on nematode parasite *O. ochengi* presented in Fig. 1 indicates that the increase in concentrations of the ethanolic extracts of *G. arborea* results in an increase in the adult mortality rate of *O. ochengi.* So, *G. arborea* leaves, barks and roots extracts induced *O. ochengi* mortality in a time and concentration-dependent manner. This anthelmintic activity of the different parts of *G. arborea* may be due to the high concentration of the great group of bioactive phytochemicals like total phenols, flavonoids and tannins in the ethanolic extracts of *G. arborea* which in contact with the worms might be toxic for them and drastically decreases the survival of the worms. Moreover, the results of the phytochemical studies presented in Table 1 reveal large quantities of the metabolites in the different parts used. It is therefore observed that the plant has a large quantity of secondary metabolites, which can provide a preliminary explanation on its filaricidal activities. Many others studies have shown that plants with anthelmintic activities contain phytochemicals such as polyphenols, tannins, flavonoids, saponins [39]. An others studies done by Fatae *et al.* [40], showed that in nematodes, tannins act on particular proteins called gluthathion s-transferase provoked their inhibitions. Klongsiriwet *et al*. [41] added that the combination of flavonoids and tannins have a synergistic anthelmintic effect against nematodes. Moreover, the drug currently used for the fight against onchocerciasis is a macrocyclic lactone derivative of avermectin-B isolated from natural source and acts as ɣ-aminobutyric acid (GABA) antagonist in nematodes. It has been extensively investigated that ivermectin acts as GABA antagonists and GluCl channel potentiators [42]. Ivermectin pass through the channels, produce the block at the narrow region and subsequently cause paralysis, immobilization and finally death of worms [43]. Therefore, it might be these phenols, flavonoids and tannins at high concentration that are responsible for the anthelmintic activities of the ethanolic extracts of *G. arborea*.

Although the different plant parts used have anthelmintic activities on *O. ochengi*, their levels of activity were not the same. Some parts had higher activity compared to others. This high activity resulted in lower lethal concentrations and the different LC_{50} of the extracts are summarized in Table 2. This higher anthelmintic activity of the roots compared to the leaves and barks may be due to the high concentration of

total phenols (873.33 ± 3.33 mg GAE/g) followed by tannins (229.33 ± 0.33 mg GAE/g) and flavonoids (116 ± 1.01 mgER/g) as revealed by the results of the phytochemical studies presented in Table 1. These secondary metabolites in contact with the worm might be toxic for them and drastically decreases the survival of the worms and a reduction of the LC_{50}. These compounds work in combination or singly to cause the mortality of these adult worms observed in this work. The LC_{50} values found in our work are similar to those obtained by Kalmobé et al. [44, 45] by studying the anthelmintic activity of the hydroethanolic extracts of the root of *Lophira lanceolata* with LC_{50} of 6.39 ± 2.11 µg/mL and of the hydroethanolic extract of the leaves and seeds of *Cucurbita pepo ovifera* with LC_{50} ranging from 17 µg/mL to 1390 µg/mL on *O. ochengi*. Dikti et al. [46] evaluated the effect of the hydroethanolic extracts of *Acacia nilotica* on *O. ochengi* males and found a LC_{50} of 11.5 µg/mL. Ndouwe et al. [47] found a LC_{50} of 2.76 ± 0.1 µg/mL with the hydroethanolic extracts of the stem bark of *Anacardium occidentale* on the same parasite as ours. Megnigueu et al. [48] with the hydroethanolic extracts of the root of *Cosmos sulphureus* and Koga et al. [49] with the methanolic extracts of the root of *Canarium schweinfurthii* on the adult worms of *O. ochengi* found, respectively, LC_{50} of 31.01 ± 1.17 µg/mL and 146.0 ± 39.0 µg/mL. A recent study done by Kalmobé *et al*. [50] revealed that the ethanolic extracts of *Aloe vera* leaves killed *O. ochengi* in a dose independent manner with LC_{50} of 21.69 ± 1.19 µg/mL.

The acute toxicity study carried out with the roots of *G. arborea* ethanolic extracts at a dose of 2000 mg/kg on the rats showed that there were no changes in the behavioral pattern and no mortality was recorded for the 14 days, observation period. So, the DL_{50} value of the tested extract was up to 2000 mg/kg, and this puts the extracts in category 5 toxicity class according to the Globally Harmonised System (GHS) of classifying substances which are regarded as relatively safe substances. These results are similar to those of Pemiah and Raj [51], who worked on the toxicity of the ethanolic extracts of the leaves of *G. arborea* on female rats. According to Kulkarni and Veeranjaneyulu [52], Bhabani et al. [53] no mortality or changes in behavioral pattern were observed after oral administration of a single dose of 2000 mg/kg of the ethanolic extract of the leaves and fruits of *G. arborea* to albino Wistar rats. In order to evaluate the long-term toxicity of *G. arborea*, it is important to evaluate the subacute toxicity with doses strictly below to 2000 mg/kg.

A 28-day oral administration was performed with the roots of *G. arborea* ethanolic extracts on rats, at the end of which, a number of parameters were evaluated to assess its toxic effects. Generally, it was reported that animals that loses 10 % of their initial body weight do not survive, which is an indication of adverse side effects of medicines, chemical products and naturals products [54, 55]. Since there was no reduction in relative body weight of the treated animals at any of the doses tested, we concluded that the extract was safe on long term administration and did not induce any alteration to metabolic system and it's considered nontoxic.

It is extremely important to assay for haematological parameters because the hematopoietic system is one of the most susceptible targets for toxic compounds. It is also used to measure the physiological and pathological status of animals and humans [56]. The main channel of food transportation nutrients and foreign bodies in the body is blood, and as such its components such as RBCs, white blood counts, platelets and hemoglobin are majorly exposed to greater dosage of toxic compounds. The resulting effects of the damages that occur to the blood cells bring about the compromise of the immune system [57]. Since our findings suggest that three doses of the ethanolic extract of the roots of *G. arborea* did not cause any significant change in the haematological parameters measured as compared to the controls in both sexes (Table 4); it indicates that the blood elements have not been damaged and that the blood's ability to transport oxygen to the tissues was preserved during this study. It could therefore be referred that the plant extract is non-toxic. Increased levels of platelets at 500 and 1000 mg/kg doses of the males in treated group compared with control group may be due to the immunostimulatory effects of the chemical compounds in the extract and also indicates that this plant could possess antianemia property. This result corroborates those of Ntchapda *et al*. [58], which showed that oral administration of *Ficus glumosa* induced the significant increase in the level of platelets at the doses of 300 and 1200 mg/kg in rats.

The liver is an imperative organ that contributes to drug biotransformation, and its normal function is monitored by serum biomarker enzymes and

protein that can be used to indicate hepatocellular effects (such as ALAT, ASAT and albumin) and the levels of creatinine and uric acid act as biomarkers of nephron functional injury [59]. They are synthesized at the level of the cytoplasm of the cells of these organs and are been discharged into the circulation when these cells are damaged [60]. Repeated oral administration of the ethanolic extracts of *G. arborea* did not caused significant change in creatinine and albumin levels in the treated batches in comparison with the control batch. Similar results were obtained by Da Silva et al. [61] with *Baccharis trimera* tincture and by Adewale et al. [62] with the aqueous extract of *Crassocephalum rubens* on rats at different doses. According to the same authors a decrease in liver enzymes ASAT and/ALAT indicated a hepatoprotective effect of the plant, which could explain the result obtained. From these observations, the administration of *G. arborea* extract is also thought to have hepatoprotective effects, thus the extract of this plant does not affect hepatic functions. According to Narayana *et al*. [63] flavonoids have been found to possess hepatoprotective and digestive protective activities consequently the presence of theses phytochemical compounds cannot permit a higher increase of ALAT and ASAT levels.

Kidneys are vital organs and highly vulnerable to toxic compounds of the body due to the large amount of blood passing through them. They filter all types of toxins which can accumulate in the tubules [64]. Urea and serum creatinine are considered to be the main markers of nephrotoxicity, although serum urea is often considered a predictor of kidney function more reliable than serum creatinine [65]. The observed result in this study suggests that the administration of *G. arborea* ethanolic extract induced a significant reduction ($P < 0.001$) in urea value in male rats treated at doses of 250, 500 and 1000 mg/kg compared to the control group. In the female rats given the same doses, the 500 mg/kg induced a significant increase in urea value ($P < 0.001$), while the 1000 mg/kg induced a significant reduction ($P < 0.001$) in the urea value. Our results are similar to those obtained by Kumaresan et al. [66] after evaluating the sub-acute toxicity of aqueous extracts of the leaves of *G. arborea* in rats. Our results are also similar to those obtained by Kanga et al. [67] and by Iteire et al. [68] in evaluating the sub-acute toxicity of aqueous extracts from the leaves of *Piper umbellatum* in rats concluded that the extracts having the capacity to decrease the level of urea is nephroprotective and does not cause renal impairment or kidney damage.

According to Zaida et al. [69], the relative weight of organs is considered as a sensitive indicator in toxicity studies. This relative weight is an indicator of organ atrophy or hypertrophy [70]. In our study, no significant changes in organ weight of the heart, spleen, kidneys and lungs was observed, except for the liver where an increase was observed at different doses (250, 500, 1000 mg/kg) in females. Thus, the ethanolic extract of the roots of *G. arborea* had no effect on the normal growth of organs nor on the morphological changes of these same organs from the batches that received the different doses of the extract of *G. arborea* compared with the organs of the rats from the control group in respective of the colour or texture. Thus, it can be suggested that the extract did not have any toxic effects. Examples of such toxic signs would be characterized by congestion, leukocyte infiltration, degeneration, necrosis, apoptosis and fibrosis in the organ tissues analyzed histologically [71]. Our results are similar to those obtained by Osseni et al. [72] who worked on the sub-acute toxicity of aqueous extracts of *G. arborea* in rats. However, the significant increase in relative weight of liver in female's rats might be due either to the low presence of flavonoids in the roots extract because these secondary metabolites are considered to be hepatoprotective or, could be due to a high sensitivity of the livers to certain bioactives compounds contained in the extract of *G. arborea*. Similar results were obtained by Jimoh et al. [73] and by Ntchapda *et al*. [74] who obtained a significant increase in the relative weight of the livers respectively after repeated administration of *Artotis arctotoides* and aqueous extracts of the leaves of *Ficus glumosa* on rats. The histopathological examination of the main organs of metabolism (liver) and elimination (kidneys) revealed that there were no signs of toxicity following a long exposure (28 days) of the extract at the doses of 250, 500, 1000 mg/kg in both sexes. The results obtained from histopathological study corroborate the claim of *G. arborea* to be non-toxic.

5. CONCLUSION

The present study assessed the hydroethanolic extract of leaves, bark and root of *G. arborea* for *in vitro* anthelmintic activity by using the cattle parasite nematode *O. ochengi*. It revealed that

the ethanolic extracts of leaves, bark and roots of *G. arborea* possesse secondary metabolites such as condensed tannins, phenols and flavonoids. These parts of *G. arborea* have anthelmintic activities on *O. ochengi* and showed a very high efficiency like ivermectin. Toxicity study shows no mortality and harmful effect in rats using different doses of the plant parts. These results have finally confirmed the efficiency of plant extracts used in traditional medicine to treat affections due to nematodes. Looking at the result, the extracts of this plant can be used as an alternative to fight against human and bovine onchocerciasis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the Animal Ethical Committee of the Ngaoundere Regional Health Authority, Cameroon.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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