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In vitro Anthelmintic Activity of Aqueous and Ethanolic Extract of *Senna italica* (Caesalpiniaceae) on Three-stages of *Haemonchus contortus*

Gilbert Yongwa[1*], Belga François Ngnodandi François Ngnodandi[1], Dieudonné Ndjonka[1] and Pierre Saotoing[2]

[1]*Department of Biological Sciences, University of Ngaoundere, P. O. BOX: 454, Ngaoundere, Cameroon.*
[2]*Department of Life and Earth Sciences, University of Maroua, P.O. BOX: 55, Maroua, Cameroon.*

Authors' contributions

This work was carried out in collaboration among all authors. Author GY designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors BFNFN and DN managed the analyses of the study. Author PS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A phytochemical screening and *in vitro* anthelminthic activity of aqueous and ethanolic extract of *Senna italica* on *Haemonchus contortus* were conducted. Polyphenol, tannin and flavonoid contents were determined by using gallic acid and rutin. egg hatching inhibition test was carried out on fresh eggs; larval mortality test was conducted on infective larvae (L_3) and adult worm mortality test was conducted. Eggs, larvae and adults worms were incubated in aqueous and ethanolic extract of *S. italica* at different concentrations (0.1; 0.3; 0.5; 0.7 and 1 mg/mL). All extracts of plants showed an effect on all stages of *H. contortus* with high efficiency variations depending on the dose used. The inhibition of eggs hatching rate increased from 8.67±1.53% to 65.67±1.15% and from 24.67±1.53% to 80±1.73% respectively for aqueous and ethanolic extract of *S. italic.* The larval mortality rate increased from 12.22±2.34% to 56.67±4.9% after 24 h and from 45.28±4.11% to 91.25±3.73% after 48 h for aqueous extract and from 30.07±2.84% to 70.83±6.31% after 24 h and

**Corresponding author: E-mail: Yongwagilbert@yahoo.fr;*

from 48.79±3.73% to 96.25±4.79% after 48 h for ethanolic extract. After 24 h of exposure to aqueous and ethanolic extract of *S. italica*, the adult mortality rate varies from 55.56±9.62% to 83.33±9.62% for aqueous extract and from 61.11±9.62% to 88.89±9.62%.These *in vitro* results confirm the use of *S. italica* in traditional medicine.

Keywords: Extract; Senna italica; activity; Haemonchus contortus; Ngaoundere; Cameroon.

1. INTRODUCTION

In Tropical Africa, breeding of small ruminants is hampered by gastro-intestinal nematodes causing the reduction of the production potential [1]. Among the disease that hinders the survival and productivity of sheep and goats, gastrointestinal nematode infection ranks highest on a global scale, with *Haemonchus contortus* being of overwhelming importance [2]. *Haemonchus contortus* is the most important nematode parasite of small ruminants, causing severe anaemia and high mortality in all classes of livestock [3]. It is one of the nematode species that dominate the parasitic spectrum of small ruminants in Africa in general and south of the Sahara in particular [4]. The infestation rate of different species of *Haemonchus* ranged from 50 - 85% [5]. The principal diagnostic feature of haemonchosis is anaemia, induced by the blood feeding nature of adults and larval stage. The average blood loss has been calculated as 0.05 mL/parasite/day [6]. Since many years, the control of haemonchosis is generally achieved by the use of synthetic anthelmintic. The frequent use of these anthelmintic over the years has inevitably led to the development of drug resistance. The emergence of resistance to anthelmintic drugs which is now a worldwide phenomenon [7] and the increasing awareness of consumers about drug residues that potentially enter the food chain have stimulated investigation into alternative anthelmintic such as medicinal plants. The treatment of gastrointestinal parasites of small ruminants by medicinal plants is a common practice in rural areas [8]. This calls into question the use of these anthelmintic. There is therefore an urgent need to seek innovative alternative solutions, in order to ensure a more sustainable control of this parasitism. Hence, new therapeutic approaches will be necessary by the use of medicinal plants, since they are accessible at all times and inexpensive [9]. The therapeutic properties of plants are strongly linked to their phytochemical components. Among a number of phytochemical constituents present in plants, saponins, polyphenols, tannins and flavonoids are kwon for their anthelmintic activity [10]. Among the therapeutics' plant, *Senna italica* family Caesalpiniaceae is known by traditional healers for the virtues of deworming animals. This plant is used in the treatment of fever; jaundice; venereal diseases and biliary crises and against intestinal worms. In South-Africa, *S. italica* is used for their antibacterial activity and efficacy against sexually transmitted disease [11]. In Cameroon, this plant is used in the treatment of hepatitis; gastroenteritis; jaundice; sexually transmitted diseases as well as stomach aches. The present study is carried out to screen for phytochemical properties and evaluate the *in vitro* activity of the aqueous and ethanolic extract of *S. italica* on *H. contortus*.

2. MATERIALS AND METHODS

2.1 Plant Material and Preparation of Extracts

The roots of *Senna italica* were collected from the Far North region of Cameroon, based on ethnopharmacological data. Voucher specimens were identified in the Department of Biological Sciences of the University of Ngaoundere. A voucher specimen was then deposited at the National Herbarium of Yaounde/Cameroon with the N°26322/SRF. The collected material was washed, dried and mashed in order to obtain a Powder. Plant extracts were prepared as described by Ndjonka et al. [12]. Briefly 100 g of powder of the plant were macerated into 1 L of ethanol at 95ºC for 48h at room temperature. The macerate was centrifuged at 3500 rpm/minute for 10 minutes and then filtered over filter papers No. 413 (VWR International, Darmstadt, Germany). The filtrate was then concentrated under reduced pressure by rotary evaporation (BUCHI Rotavapor R-200, Switzerland) at 40°C. Residual solvent was removed by drying in a sweating-room at 35°C and the extract was weighed and stored at + 4°C. The plant extract was further dissolved in dimethyl sulfoxide (DMSO) and phosphate buffer solution (PBS) to a final concentration of 100 mg/ml, centrifuged and aliquoted to determine their *in vivo* activity on *H. contortus*.

2.2 Phytochemical Analysis

The overall phenolic content was assessed using Folin-Ciocalteu reagent [13]. The absorbance was measured at 765 nm using a spectrophotometer and the results were expressed as mg of gallic acid equivalents (GAE) per gram of extract (mg GAE/g). The flavonoid content was determined by applying aluminum chloride colorimetric method described by Barros et al. [14] and expressed as mg of rutin equivalents (RE) per gram of extract (mg RE/g). The condensed tannin content was analyzed by using the vanillin assay described by Ba et al. [15]. The results were expressed as mg of catechin equivalents (CE) per gram of extract (mg CE/g). The saponin constituent was quantified according to the modified method described by Jamuna et al. [16]. A 0.01 g of extract was added to 10 ml of distilled water and the mixture was vigorously shaken for 30 mins. The height of moss was measured by using a graduated ruler and quantified according to the following formula:

Saponin (mg/g) = [(0.432) *(height of moss in cm after 15 s) + 0.008] / (weight of extract in gram).

2.3 Tests on *Haemonchus contortus*

2.3.1 Collection of adults of *Haemonchus contortus*

The abomasa of goats and sheep were bought from markets in Ngaoundere. These abomasa were transported to the laboratory of Zoology of the University of Ngaoundere. Using the protocol described by Kabore [8] for obtaining females adults of *H. contortus* based on the presence of the vulva. The abomasa were incised in order to collect all worms and transferred in PBS solution. Females of *H. contortus* were isolated under binocular microscope at objective ×10.

2.3.2 Anthelmintic bioassay on adults *Haemonchus contortus*

Solutions of the ethanolic extract of roots of *S. italica* were prepared with PBS for 04 concentrations (0.04; 0.06; 0.08 and 0.1 mg/ml). Worms which presented good mobility were used for this test. One worm was introduced in well containing 500 µL of solution for 03 columns of 06 wells. The PBS was used as negative control and a positive control consisted of albendazole. The test solution were concomitantly prepared and incubated at 37°C for 24h. Worm mortality was checked by observing under the binocular microscope. After shaking worm, immotile and fully elongated worms were considered to be dead. The mortality rate was determined after 24 h. The mortality rate was calculated using the formula below:

$$\text{Mortality rate} = ND/NT \times 100$$

Where ND is the number of dead worms in each well and each concentration. NT is the total number of worms in each well and in each concentration.

2.4 Tests on Eggs of *Haemonchus contortus*

2.4.1 Parasite donor goat

The abomasa of goats were obtained from the abattoir of the “Marché bantay” of Ngaoundere town after necropsy of small ruminants. Adult female worms were identified using morphometric and morphologic characteristics according to Ahmed et al. [17]. After collecting female *H. contortus*, they were crushed to liberate eggs [18]. Eggs obtained were cultured in petri dishes at room temperature for seven days [19]. The culture media constituted of 3 ml of sterile liquid of feaces prepared from 3 g of faeces removed from the rectum of parasites free goat, to which was added charcoal. At the end of the 8th day, infective larvae were harvested. About 4500 larvae were estimated by counting the number of larvae contained in 0.1 mL of well homogenized solution of infective larvae. After five repetitions of counting, the mean number of larvae in 0.1 mL of solution was determined and the volume containing 2500 larvae were deduced, measured and inoculated into a worm-free goat. This goat served as *H. contortus* egg donor for the *in vitro* trials.

2.4.2 Recovery of nematode eggs

After the pre-patent period of 21 days, 3 g of faeces were collected directly collected from the rectum of the donor goat. According to the procedure described by Wabo et al. [20], faeces were homogenized in a mortar by adding 60 mL of salt (Na Cl 40% W/V). The solution was cleaned of organic debris by filtration through a 250 µm mesh-size sieve into a beaker and finally poured into four conical tubes until the formation of a meniscus at the top. Three minutes later, slides and cover slides containing the eggs were

rinsed with distilled water into 100 mL beaker. The beaker was allowed to stand for 30 minutes for the sedimentation of the eggs at the bottom. To completely remove the salt solution, eggs were washed three times by siphoning out 90 mL of solution and replacing with the same amount of distilled water each 30 minutes. Finally the supernatant was removed and the remaining solution containing eggs was used in the assay.

2.4.3 Egg Hatch Assay (EHA)

The *in vitro* EHA was based on the method described by Coles et al. [21]. Fresh eggs of *H. contortus* were used to evaluate the ovicidal activity of aqueous and ethanolic extract of *S. italica.* To do this, 30 eggs of *H. contortus* were distributed into each well of a 24-flat-bottomed microtitre plate and incubated in different concentration (0.1; 0.3; 0.5; 0.7 and 1 mg/mL) of aqueous and ethanolic extract of *S. italica*. Albendazole was used as positive control and evaluated at various concentrations (0.1; 0.3; 0.5; 0.7 and 1 mg/mL). PBS was used as negative control. The plates were incubated for 48 h at 27 °C. The experiment was replicated three times for each extract on the same plate. After incubation, the hatched larvae and unhatched eggs were counted using an inverted microscope under 20 × magnifications. The percentage Egg Hatching Inhibition (%EHI) was calculated using the formula:

$$\%\text{EHI} = 100 - (\frac{\text{Number of L1 larvae}}{\text{Number of fresh eggs in culture}} \times 100$$

2.5 Tests on Infective Larvae of *Haemonchus contortus*

2.5.1 Recovery of nematode larvae

The infective larvae were obtained by stool culture from feces of goats (goat donor) previously infested by *H. contortus* larvae. The feces were collected directly from the goat's rectum. The eggs contained in the fecal matter were subsequently placed in stool culture at room temperature for 7 days. At the end of this culture time, the larvae were extracted from the faecal mass by the Baermann device, the principle of which is based on the hygrotropism of the larvae.

2.5.2 Evaluation of larvicidal activity of *Senna italica*

Larval mortality assay using L_3 larvae was performed according to the method described by Wabo pone et al. [20]. Aqueous and ethanolic extract of *Senna italic* and positive control made by albendazole were dissolved in PBS. Twenty larvae were distributed into each well of a 24-flat-bottomed microtitre plate at different concentration (0.1; 0.3; 0.5; 0.7 and 1 mg/mL). PBS was used as negative control. The plates were incubated for 24 and 48 h at 27°C. The experiment was replicated three times for each extract on the same plate. After the incubation's period, the number of dead larvae was counted under the microscope based on their stranght shaped, their immobility and the presence of holes. The percentage of mortality (Mt%) was determined using the following formula:

$$\text{Mt}\ (\%) = (\frac{\text{Number of dead larvae}}{\text{Number of larvae in culture}}) \times 100$$

2.6 Statistical Analysis

The 50% inhibitory concentrations (IC_{50}) for eggs hatching rates were calculated using linear regression equations drawn after transformation of the eggs hatching inhibition rate to probit according to the decimal logarithm of concentrations. While the 50% lethal concentrations (LC_{50}) for L_3 larvae was determined using linear regression equations drawn after transformation of larval mortality rate to probit according to the decimal logarithm of concentrations. Comparison of the mean inhibition percentage of eggs hatching and mean percentage of larval mortality at different concentrations with control was performed by two-way analysis of variance (ANOVA). Statistical analyses were performed using the software SPSS version 17.0 software. The post hoc statistical significance test employed was LSD, differences between the means were considered significant at $P < 0.05$. The 50% inhibitory concentration (IC_{50}) and lethal concentration (LC_{50}) were determined.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

The quantification of phytochemical metabolites of aqueous and ethanolic of roots of *S. italica* was carried out to evaluate the chemical families present in the plant extracts and which might be involved in the anthelmintic activity. Polyphenols; flavonoids, tannins and saponins were quantified; the results of these assays are presented in Table 1. It appear from this table that

polyphenols and tannins content in aqueous and ethanolic extracts are highest compared to flavonoids and saponins. The Table 1 also revealed that saponins are absent in both extracts of the plant. In a study performed by Barbosa et al. [22] the same compounds have been found in roots, seeds, bark and leaves of *S. italica* and described as the main active ingredient. Dabai et al. [23] have found in leaves of *Senna rugosa,* alkaloids; steroids and flavonoids but noted the absence of tannins in all the different extracts of the plant. These differences would be due to certain factors such as the harvest season and geographical location. According to Adoum et al. [24], the geographical location of plants can affect its bioactive constituents induced by factors such as climate and soil. In a study conducted by Rajesh et al. [10], the activity of a plant extract depends on the availability of secondary metabolites like tannins; saponins; polyphenols; triterpens and flavonoids. The aqueous and ethanolic extract of roots of *S. italica* contains almost the same secondary metabolites namely polyphenols; flavonoids and tannins which might be toxic for the worms and responsible for the observed anthelmintic activity.

3.2 Anthelmintic Activity of *Senna italica* on Adults *Haemonchus contortus*

The *in vitro* study is a way to evaluate the anthelmintic activity of the aqueous and ethanolic extracts of *S. italica* on adults *H. contortus* (Table 2). Based on the present results, it appears that the increase in concentrations of albendazole as well as aqueous and ethanolic extracts of the root of *S. italica* results in an increase in the adult mortality rate of *H. contortus* in a concentration-dependent manner. In fact, albendazole has significantly induced ($p < 0.05$) mortality of *H. contortus* compared to negative control (PBS) with mortality rates of 77.78% 100% with a LC_{50} of 0.059±0.025 after 24 h incubation. The mortality of adult worms of *H. contortus* has been induced *in vitro* by the aqueous and ethanolic extracts of *S. italica* with a mortality rate ranging from 55.56% to 83.33% with the LC_{50} 0.079±0.03 and from 61.11% to 88.89% with the LC_{50} 0.089±0.04 respectively for aqueous and ethanolic extracts. Similar results were also reported by Dedehou et al. [25] with *Erocarpus erinaceus* extracts and *Parkia biglobosa* fruit pods on *H. contortus*. These authors obtained a

Table 1. Phytochimical screening of aqueous and ethanolic extract of *Senna italica*

AES	Polyphenols	Flavonoids	Tannins	Saponins
	43.67±0.56	11.56±0.43	16.76±0.06	0±00
EES	46.67±0.63	10.56±0.23	13.21±0.01	0±00

AES: aqueous extract of S. italica; EES: ethanolic extract of S. italica

Table 2. Effect of *Senna italica* root extract on female *Haemonchus contortus*

	Concentrations (mg/mL)	Mortality of females *H. contortus*
PBS	0	0
EES	0.1	61.11±9.62
	0.3	66.67±0
	0.5	83.33±0
	0.7	83.33±0
	1	88.89±9.62
AES	0.1	55.56±9.62
	0.3	66.67±0
	0.5	77.78±9.62
	0.7	77.78±9.62
	1	83.33±9.62
Albendazole	0.1	77.78±9.16
	0.3	94.44±9.65
	0.5	100±00
	0.7	100±00
	1	100±00

AES: aqueous extract of S. italic; EES: ethanolic extract of S. italica

maximum mortality rate of 100% after 24 h of incubation in the hydro-acetonic extract at the concentration of 1200 µg/mL. Similar observation were also noted by Hounzangbe-Adote et al. [26] with the alcoholic extracts of four Benin plants, Zanthoxylum zanthoxyloides, *Newbouldia laevis*, *Morinda lucida* and *Carica papaya*.

3.3 Anthelmintic Activity of *Senna italica* on Egg Hatching Inhibition (EHI) of *Haemonchus contortus*

Table 3 presents the variation of the mean EHI of *H. contortus* at different concentrations. It appears from these results that increased concentrations of albendazole as well as aqueous and ethanolic extracts of *S. italica* result in an increase in the rate of inhibition of egg hatching. In fact, albendazole has significantly inhibited the embryonation of the eggs with mean inhibition rate of 27.68±2.52 to 100±00 with a LC_{50} of 0.269 ± 0.005 mg/mL ($p <0.05$). While aqueous and ethanolic extract of *S. Italica* inhibited the embryonation of eggs from 8.67±1.53 to 65.67±1.15 and from 24.67±1.53 to 80±1.73 with a LC_{50} of 0.69±0.03 mg/mL and 0.48±0.01 mg/mL respectively for aqueous and ethanolic extract ($p <0.05$). The present findings results corroborate with those of Wabo et al. [19] who achieved anthelmintic efficacy on EHI with bark extract of *Canthium mannii* stems on *Ancylostoma caninum* eggs. In South Africa, Fouche et al. [27] obtained 55% EHI of *H. contortus* with the acetonic extract of *S. italica*. Pessoa et al. [28] achieved a total inhibition of egg hatch 2.5 mg/mL concentration in their *in vitro* tests with *Ocinum gratissimum* essential oil on *H. contortus* eggs. Similarly, Costa et al. [29] achieved 91% efficiency in EHI of eggs *H. contortus* with the ethanolic extract of *Mangifera indica* at a concentration of 10 mg / mL. In contrast, aqueous extracts of *Annona senegalensis* seeds inhibited *H. contortus* egg hatch by only 11% at a concentration of 7.1 mg/mL [30]. The LC_{50} values obtained in present study are nearly similar to those of Eguale *et al.* [18] with LC_{50}s of 0.87, 0.10 and 0.06 mg / mL with aqueous extracts of *A. nilotica, C. macrostachyus* and *E. capensis* respectively and lesser to those of Maciel et al. [31] with a LC_{50} of 2.2 mg / mL with ethanolic extracts of *M. azedarach* on the inhibition of eggs hatching of *H. contortus*.

3.4 Anthelmintic Activity of *Senna italica* on Mortality of L_3 of *Haemonchus contortus*

Table 4 shows the effects of different extracts of *S. italica* on L_3 larvae of *H. contortus* after 24 and 48 hours. The aqueous and ethanolic extracts of *S. italica* induced paralysis of the infective larvae (L3), depending on concentrations and time. It was observed that a significant increase in the rate of paralysis of larvae L_3 *H. contortus* occurred at dose (1 mg / mL) after 24 and 48 h of incubation. In fact, the aqueous and ethanolic extract of *S. italica* as well as albendazole induced paralysis of infective larvae in the range of 12.22 ± 2.34 to 56.67 ± 4.9 and 30.07 ± 2.84 at 70.83 ± 6.31 respectively for the aqueous and ethanolic extract, with LC_{50} of 0.85 ± 0.02 and 0.62 ± 0.05 after 24 h respectively.

Table 3. Effect of *Senna italica* root extract on *Haemonchus contortus* eggs hatching inhibition

	Concentrations (mg/mL)	Egg hatching inhibition (EHI)
PBS	0	3.5±1.2
EES	0.1	24.67±1.53
	0.3	41.67±1.54
	0.5	60±4.36
	0.7	70±1
	1	80±1.73
AES	0.1	8.67±1.53
	0.3	24±1
	0.5	40±3
	0.7	49.333±0.58
	1	65.67±1.15
Albendazole	0.1	27.68±2.52
	0.3	63.67±4.16
	0.5	86±2.65
	0.7	100±00
	1	100±00

AES: aqueous extract of S. italica; EES: ethanolic extract of S. italica

Table 4. Larvicidal activity of *Senna italica*

	Concentrations (mg/mL)	24 h	48 h
AES	0.1	12.22±2.34	45.28±4.11
	0.3	19.78±3.67	57.78±2.52
	0.5	27.11±4.22	58.335±3.85
	0.7	32.5±5	59.585±8.43
	1	56.67±4.9	91.25±3.73
EES	0.1	30.07±2.84	48.79±3.73
	0.3	32.57±3.22	51.29±1.61
	0.5	61.01±5.71	75.59±7.44
	0.7	63.09±9.38	79.76±4.26
	1	70.83±6.31	96.25±4.79
Albendazole	0.1	52.20±9.86	80.43±3.99
	0.3	54.37±5.15	89.37±5.91
	0.5	78.03±5.14	100±00
	0.7	92.25±9.67	100±00
	1	100±00	100±00

AES: aqueous extract of S. italica; EES: ethanolic extract of S. italica

After 48 h, the anthelmintic efficacy was greater with a mortality rate varying between 45.28 ± 4.11 at 91.25 ± 3.73 and 48.79 ± 3.73 at 96.25 ± 4.79 respectively with the aqueous and ethanolic extract for LC_{50}s less than those obtained 24 hours after contact are respectively 0.44 ± 0.06 and 0.31 ± 0.05 for the aqueous and ethanolic extract of *S. italica*. These results corroborated with those of Okombe [32] with a L_3 paralysis inhibition of 85.61% at 2 mg / mL concentration with ethanolic extracts of *Vitex thomasii* root bark powder and 71.11% with aqueous extracts from the same plant. Albendazole, a synthetic anthelmintic used as a positive control in our *in vitro* tests is a commonly used drug whose mechanism of action has been demonstrated by many researchers. Alvarez et al. [33] have highlighted two main mechanisms that cause the destruction of nematode parasites. The first is the diffusion of the anthelmintic product through the outer surfaces such as the eggshell and larval cuticles and the second, diffusion through the intestinal cells.

According to several authors [30,34,35], the anthelmintic activity of plants is attributed to the secondary metabolites present in plants respectively polyphenols; flavonoids and tannins. To this end these plants metabolites may have work in combination or singly to cause inhibition of egg hatch, larval mortality and adult mortality that was observed in this work.

Indeed, the secondary metabolites present in the different plant extracts used in this study could have attached on eggs, larvae or adults and cause the desired effects. The effectiveness of plant extracts on both egg hatch inhibition and larval paralysis and adult death may be easier to diffuse through egg shells and cuticles of larvae and adults worms. According to Athanasiadou et al. [36], the secondary metabolites of plant extracts could bind to available free proteins to render them unusable as nutrients and cause larval death in eggs or mortality of larvae and adults. Indeed, the important anthelmintic effect obtained with extracts of *S. italica* in our study would probably be due to the fact that it is more concentrated in active principles.

The findings of this present study showed that aqueous and ethanolic extract of *S. italica* exhibited evidence of *in vitro* anthelmintic activity against eggs larvae and females adults of *H. contortus.*

4. CONCLUSION

In conclusion, this work focused on the *in vitro* activity of the aqueous and ethanolic extracts of *Senna italica* on *Haemonchus contortus.* It appears from the results of the present study that aqueous and ethanolic extract of *S. italica* exhibited evidence of *in vitro* anthelmintic activity on eggs; infective larvae L_3 and against adults of *H. contortus*. However, further *in vivo* studies are required to evaluate the bioactivity of the extracts of the above plant and to investigate the potential presence of toxic effects in order to determine the minimum non-lethal doses for the treatment of gastrointestinal helminths.

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.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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