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In vitro Anthelmintic Activity of *Tephrosia pedicellata* on Two Nematodes (*Haemonchus contortus, Caenorhabditis elegans*) and Its *Ins vivo* Toxicity on Rats

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Abstract: In Cameroon, the practice of traditional medicine in the treatment of intestinal helminthiasis is a reality and habits in the lives of traditional healers and breeders for various reasons related to their local life situations. In the valorization of medicinal plants in the treatment of digestive parasitosis as an alternative to synthetic anthelmintics, a study was carried out in order to verify their anthelmintic activity and the level of their toxicity. The present study was carried out to determine the *in vitro* anthelminthic activity of *Tephrosia pedicellata* extracts. Aqueous and hydroethanolic leaves extracts at different concentrations were tested against *Haemonchus contortus* development stages, mutant and wild type of free-living nematode *Caenorhabditis elegans*. The two plant extracts were then chemically screened and their toxicological profile was established using albino rats. Hydroethanolic extract was the most potent by killing *Haemonchus contortus* adults (LC_{50}: 0.038 mg/mL after 24 hours), infesting larvae (LC_{50}: 0.22 mg/mL after 48 hours) and inhibiting egg hatching (IC_{50}: 0.76 mg/mL after 48 hours). The two plant extracts showed same activity on *Caenorhabditis elegans* levamisole non-sensitive (LC_{50}: 0.26 mg/mL after 48 hours) and sensitive strains (LC_{50}: 0.25 mg/mL after 48 hours), showing different mode of action to that of levamisole. Several plant chemicals were identified from both extracts, with higher content of polyphenol compounds (562.91 mg GAE/g DW), flavonoids (135.96 mg RE/g DW), condensed tannins (228.50 mg CE/g DW) and saponins (206.83 mg SE/g DW) obtained with the hydroethanolic extract. No toxic effect was observed during acute toxicity study. Moreover, apart from the significant increase of platelets and Aspartate transaminase (p=0.001) and the glomerular inflammation in the lungs at the dose of 1000 mg/kg, no harmful variation of haematological and biochemical parameters of rats were observed. The pattern of anthelmintic activity of these extracts on *Caenorhabditis elegans* and the non-toxic effect of hydroethanolic extract provide a way for new anthelminthic drug.

Keywords: *Haemonchus contortus*, *Caenorhabditis elegans*, *Tephrosia pedicellata*, Plant Chemicals, Anthelminthic, Toxicity

1. Introduction

Livestock is an important sector of foresight that can help solve the problems of small farmers and thus help fight against poverty [1]. Despite the steps under taken by some countries, livestock farming faces serious problems of pathological order such as bacterial, viral and parasitic diseases. Among these pathologies, parasitic diseases occupy a prominent place because of the losses they cause on animal productivity [2]. Among these parasitic diseases, haemonchosis is the most significant, with high impact on the productivity of small ruminants with a prevalence rate varying between 95 and 98%

in some regions [3]. This parasite, is able to cause symptoms such as inflammation and bleeding of the mucosa, digestive disorders with diarrhea, weight loss, impaired quality of wool, impaired reproductive capacity [4], loss of appetite, edema, deterioration of the animal general condition, deficiency in certain vitamins [5], but also mortality from severe anemia. The fight against these intestinal helminthiases has been based on the use of synthetic anthelmintics belonging to the families of Benzimidazoles, Imidazothiazoles and Macrocyclic lactones. However, there are several problems with their uses. In many developing countries, small farmers have difficulty accessing veterinary medicines [6]. Added to this are the high cost of products in health facilities, the strong dependence on the outside for the supply of essential drugs, the low level of medical coverage and the toxicity of these synthetic products [7]. Moreover, the excessive use of synthetic anthelmintics has led to the emergence of resistance worldwide [8, 9].

Caenorhabditis elegans is a free living nematode introduced in the genetic laboratories in the early 1960s by Brenner as a model for studying developmental biology and functioning of the nervous system [10]. Indeed, the general simplicity of this organism, its ease of breeding, its transparency, its short life cycle and its ability to generate a multitude of descendants in a simple *in vitro* culture medium make it a very good study model [11]. The use of *C. elegans* as a model for the study of parasitic nematodes has increased since it was used in 1981 to select potential anthelmintic compounds [12]. Phylogenetic analysis of the phylum Nematoda suggests that Trichostrongles (*H. contortus*) are more closely related to *C. elegans* [13]. Moreover, *C. elegans* belongs to the order Rhabditina and included in the same phylogenetic clade as *H. contortus* and other parasites of human and veterinary importance.

Given the problems associated with synthetic anthelmintics, the use of medicinal plants containing secondary metabolites such as phenolic compounds and saponins known for their anthelmintic activity [14], could be an important alternative in the fight against gastrointestinal strongyles of small ruminants. However, although medicinal plants have several therapeutic virtues, they are not exempt from all danger of intoxication. It is therefore important to check the level of toxicity of these plants. *Tephrosia* genus belongs to the family of Fabaceae with subfamily Paplilionaceae. This genus contains more than 350 species. The plants from this genus are mainly found in the tropical, subtropical, and arid regions of the world [15]. *Tephrosia pedicellata* is an herbaceous plant with a more or less perennial base and annual stems. In traditional medicine *T. pedicellata* is used in the treatment of helminthiasis, naso-pharyngeal infection and to treat diseases affecting the nervous system [16]. The objectives of this study are to investigate the *in vitro* anthelmintic activity of *T. pedicellata* aqueous and hydroethanolic extracts on three stages of *H. contortus* and the free-living nematode *C. elegans*. In addition, to study the phytochemical composition, the acute and subacute toxicity of these extracts on rats.

2. Materials and Methods

2.1. Plant Materials

Leaves of *T. pedicellata* were harvested in May 2017 in the Far-North Region, Cameroon and the plant was identified by Prof. Pierre Marie Mapongmetsem and Dr. Fawa Guidawa, from the Department of Biological Sciences, University of Ngaoundere, Cameroon and confirmed at the Cameroon National Herbarium (Yaoundé) by TADJOUTEU Fulbert. The corresponding reference number of identification is 67001/HNC. These leaves were thoroughly washed in water and left to dry at room temperature for about 15 days. The dried leaves were ground to powder with a mortar and pestle and stored at room temperature in airtight jar till usage.

2.2. Preparation of Plant Extracts

A hydroethanolic and aqueous extracts were prepared according to the protocol of Ndjonka *et al*. [17]. Fifty grams of powdered material was extracted with 500 mL of 70% ethanol or distilled water for 48 hours for the hydroethanolic and aqueous extract respectively, centrifuged (3500 g, 10 min) and filtered through filter paper n° 413 (VWR International, Darmstadt, Germany). The clear filtrate was concentrated by means of a rotary evaporator (BUCHI Labortechnik AG, Switzerland) at a temperature of 40°C under reduced pressure of 175 mbar, lyophilized at -60°C with a vacuum of 1 mm Hg for 48 hours and the resulting powder was stored at 4°C.

2.3. Phytochemical Study

2.3.1. Qualitative Phytochemical Tests

Qualitative phytochemical screening was carried out with both aqueous and hydroethanolic extracts using the standard method based on coloring and precipitation reactions. These extracts were used to test for the presence of flavonoids, leucoanthocyanins, terpenoids and steroids according to the method of Fankam *et al*. [18]; alkaloids using the method of Lerato *et al*. [19], tannins according to the Bargah method [20]; saponins by the method of Dohou *et al*. [21] and cardiac glycosides by the method of Edeaga [22].

2.3.2. Quantitative Determination of the Chemical Components

The quantitative analysis was aimed to determine the phytochemical content of the extracts. Total phenolics, flavonoids, tannins and saponins were measured using colorimetric methods. All quantitative analyzes were performed in triplicate. Total phenolic compounds were determined according to the Folin-Ciocalteu (FC) method used by Boizot and Charpentier [23]. The total flavonoid contents were measured according to the colorimetric test described by Bahorun *et al*. [24]. The amount of condensed tannins was determined according to the method of vanillin in an acidic medium as described by Ba *et al*. [25]. The total saponin content of the various extracts was determined by the vanillin-sulfuric acid method described by Hiai *et al*. [26].

2.4. Animal Materials

Adults *H. contortus* were obtained from the abomasum of goats and sheep collected at the small ruminant slaughterhouse in Ngaoundere (Cameroon) after autopsy of the animals. Fecal material from a donor goat experimentally infected with *H. contortus* was collected to obtain *H. contortus* eggs and L3 larvae. Levamisole-resistant (CB211) and wild-type strains of *C. elegans* were purchased from the Caenorhabditis Genetics Center (Minneapolis, Minnesota, USA) and multiplied according to the method of Ndjonka *et al*. [17]. Wistar rats were provided by the National Veterinary Laboratory of Garoua, Cameroon.

2.5. Collection of Adults of Haemonchus contortus

Goat and sheep abomasum were collected immediately after slaughter and transported to the laboratory of the University of Ngaoundere. Once in the laboratory, the abomasa were washed with tap water and the parasites were stored in phosphate buffered saline (PBS) until the start of *in vitro* evaluation. The collected worms were placed under the dissecting microscope to identify the adult females of *H. contortus* recognized by the presence of the vulvar flap.

2.6. In vitro Anthelmintic Activity on Haemonchus contortus

2.6.1. Adult Mortality Test

The anthelmintic activity of aqueous and hydroethanolic extracts of *T. pedicellata* leaves on adults of *H. contortus* was studied according to the method of Dedehou *et al*. [27] with slight modifications. The test was performed in a 24-well plate. Six different concentrations in PBS (phosphate buffered saline) (0.05; 0.2; 0.4; 0.6; 0.8 and 1 mg/mL) of plant extracts were used to assess their anthelmintic potential against adults of *H. contortus*. Six healthy and active parasites were incubated in each concentration. Levamisole at the same concentrations was used as a positive control and PBS as a negative control. The worms were incubated at 37°C and the number of motile (live) and immotile (dead) worms was counted every 12 hours for 24 hours under a stereomicroscope and recorded for each concentration. Death worms were noted by lack of motility even after shaking the plates. The experiment was performed in triplicate for each treatment. The percentage of adult worm mortality was determined using the following formula:

$$\%\ \text{Adult mortality} = \frac{\text{Number of dead worms}}{\text{Number of worms in culture}} \times 100 \qquad (1)$$

2.6.2. Egg Hatch Inhibition Test

Haemonchus contortus eggs were recovered according to the method described by Beaumont *et al*. [28]. Briefly, about 40 g of fecal matter obtained from a donor goat was suspended in 100 mL of water using an electric mixer. This suspension was filtered through a sieve. The filtrate was centrifuged at 1500 rpm for 5 min and the supernatant was removed, replaced with NaCl solution (density: 1.2), mixed and centrifuged at 1000 rpm for 5 min. The eggs were collected in the upper meniscus of the tube. The eggs were washed 3 times with distilled water while centrifuging at 1000 rpm for 5 min. The amount was adjusted to reach a concentration of 50 eggs/500 µL. The number of eggs was estimated by the McMaster technique [29]. Six increasing concentrations of the aqueous and hydroethanolic extracts were prepared: 0.125; 0.25; 0.5; 1; 2 and 4 mg/mL diluted in PBS. 0.5 mL of suspension corresponding to 50 eggs was placed in each well of the 24-well plate and mixed with 0.5 mL of plant extract at the corresponding concentrations. A negative control (PBS) and a positive control (levamisole at the same concentrations) were involved. The plates were incubated for 48 hours at 27°C under vacuum and after incubation a drop of formaldehyde solution (10%) was added to prevent the eggs from hatching. All eggs and first instar larvae (L1) in each plate were counted under a stereomicroscope. There were three repetitions for each focus and control. Data were expressed as a percentage of unhatched eggs. The eggs hatch inhibition (EHI) were calculated by the following formula:

$$\%\ \text{EHI} = \frac{\text{Total number of eggs} - \text{number hatched larvae}}{\text{Total number of eggs}} \times 100 \quad (2)$$

2.6.3. Larval Mortality Test

The larval mortality test was carried out as described previously by Martinez *et al*. [30]. Larvae were obtained by larval culture from goat faeces previously artificially infected with strains of *H. contortus*, incubated at room temperature for 10 days to obtain L3 larvae. The larvae were then extracted from the fecal mass using the Baermann method, which uses the hygrotropism of the larvae. A known quantity of infesting larvae (50/500 µL) was added to 0.5 mL of plant extract at different concentrations (0.15; 0.31; 0.62; 1.25 and 2.5 mg/mL) and incubated for 24 and 48 hours at 25°C. As a negative control, PBS was used and levamisole as a positive control (at the same concentrations with plant extracts). There were three replicates for each concentration of extract and control. Examination of all larvae in each well was done under a stereomicroscope to count the number of larvae that were dead or alive. After the incubation time, 10 µL of an enzymatic dye: MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the larvae for 3 hours. The advantage of this step is that the MTT will cause enzymatic reactions which stain the living larvae blue-violet. Dead larvae will remain colorless. The count of paralyzed larvae appearing coiled up was carried without difficulty. The percent mortality of the larvae was determined using the following formula:

$$\%\ \text{Larval mortality} = \frac{\text{Number of dead larvae}}{\text{Number of larvae in culture}} \times 100 \quad (3)$$

2.7. Mortality Assay of Adult Caenorhabditis elegans Mutant Strains (CB211) and Wild Type (WT)

The test on adult *C. elegans* was carried out according to the procedure of Katiki *et al*. [31]. The test was performed with young adults with intact cuticles. The solutions to be tested were prepared in the M9 buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 0.25 g MgSO4.7H2O, in 1 liter of water) in six different concentrations (0.25; 0.5; 0.75; 1; 1.25 and 1.5 mg/mL). The test was performed using a 24 well plates and a number of worms (adjusted to 15 worms) was deposited in each well and placed in

contact with each concentration to a final volume of 500 µL/well. The negative control was performed with M9 buffer. Three tests were performed for each concentration. The plates were then incubated at 20 °C under vacuum for 24 and 48 hours. After incubation, the plates were read using a stereomicroscope and all adult worms were counted and determined to be dead or alive, according to [32]. They were considered dead when they showed no movement and as alive when there were at least a few movements of the tail, head or pharynx (for 30 seconds of observation). The percentage of worm mortality was determined using the following formula:

$$\% \text{ mortality} = \frac{\text{Number of dead worms}}{\text{Number of worms in culture}} \times 100 \qquad (4)$$

2.8. Assessment of Acute and Subacute Toxicity of Extract of Tephrosia pedicellata

2.8.1. Acute Toxicity

The acute toxicity test was performed in accordance with OECD Test Guideline 423 [33]. Fifteen nulliparous, non-pregnant and normal female rats were randomly selected and divided into five groups of three rats each. They were then kept at room temperature for 5 days, under experimental acclimatization conditions in the animal facility of the Department of Biological Sciences of the University of Ngaoundere. All groups of rats were fasted overnight and weighed before oral gavage according to their body weight. The hydroethanolic extract of *T. pedicellata* leaves was then administered orally at a dose of 5 mg/kg (group 2), 50 mg/kg (group 3), 300 mg/kg (group 4) and at the limit dose of 2000 mg/kg (group 5) of body weight of rats. The control group (group 1) received only distilled water (10 mL/kg). After administration, the rats were observed continuously for the first four hours and daily for 14 days for signs of toxicity namely, changes in skin, hair, motor activity and behavior. Much attention was paid on the following manifestations, tremors, sensitivity to noise, convulsions, salivation, diarrhea, lethargy, sleep and coma.

2.8.2. Subacute Toxicity

The study was conducted in accordance with OECD Guideline 407 [34]. Briefly, twenty-four rats were randomly assigned to four groups (1, 2, 3 and 4) of six rats each (three females and three males). The rats of group 1 corresponding to the control group received distilled water (10 ml/kg). While the hydroethanolic extract of *T. pedicellata* at doses of 250, 500 and 1000 mg/kg body weight was administered orally to groups 2 to 4 respectively, every day for 28 days, and their weight was measured every week for four weeks. During this time, the animals were carefully examined daily for signs of toxicity. On day 28, the rats were sacrificed and blood samples were taken from the jugular vein during the animal euthanasia procedure. Blood samples were taken in tubes containing ethylene diaminotetraacetic acid (EDTA) and non-heparinized tubes for analyzes of hematological and biochemical parameters, respectively. Hematological parameters were analyzed using an automated hematology analyzer (Coulter, Mindray). Tubes containing blood without anticoagulant were centrifuged at 4000 rpm for 10 min and the resulting serum was stored at -20 °C for blood biochemical analyzes. Several biochemical parameters were measured, in particular serum urea by the UV urease-GLDH kinetic method, using the UREA UV SGM-italia kit; creatinine by the Jaffé colorimetric method, using the CREATININE LR SGM-italia kit; alanine aminotransferase (ALT) by the IFCC optimized UV kinetic method, using the GPT-ALT LR GSM-italia kit; aspartate aminotransferase (AST) by the IFCC optimized UV kinetics method, using the GOT-AST LR GSM-italia kit and glucose by the ENDPOINT enzymatic colorimetric method, using the Cromatest Linear Chemicals kit. Histological analyzes were carried out according to the protocol of John and Alan [35]. At the end of the treatment period, the major organs (liver, kidneys and lungs) were removed and carefully fixed in 10% formalin. After fixation, the tissue samples were dehydrated to increasing concentrations of ethanol (70-100%), embedded in paraffin and cut into thick sections. Tissue samples were stained with hematoxylin and eosin for microscopic observation at 40x (lungs), 100x (liver) and 200x (kidney) magnifications, using a light microscope (ZEISS Axioskop brand), equipped with a photomicrography device connected to computer.

2.9. Statistical Analyses

Results were summarized as means±standard error of mean (n=3). The extract concentration required to inhibit egg hatching by 50% (IC_{50}) and the lethal concentration 50 (LC_{50}) for larval and adult mortality was calculated using probit analysis. The effect of plant extracts and that of levamisole were subjected to ANOVA (bidirectional analysis), following post hoc testing by the Tukey and Dunnett procedures. A significance level of 5% was used and the analysis was performed by the software XLSTAT.Premium.v2016.02.28451.

3. Results

3.1. Qualitative Phytochemical Analysis

The phytochemical characterization tests carried out on the aqueous and hydroethanolic extracts of *T. pedicellata* leaves are presented in Table 1. Phytochemical screening shows that the aqueous extract of *T. pedicellata* leaves contain polyphenols, flavonoids, tannins, saponins, alkaloids and terpenoids. Same results were obtained with the hydroethanolic extract except for the presence of cardiac glycosides. Steroids and leucoanthocyans were absent in both extracts.

Table 1. *Preliminary phytochemical screening of aqueous and ethanolic extracts of Tephrosia pedicellata leaves.*

Components	Aqueous extract	Hydroethanolic extract
Polyphenols	+	+
Flavonoids	+	+
Tannins	+	+
Saponins	+	+
Alcaloids	+	+
Terpenoids	+	+
Steroids	-	-
Leucoanthocyans	-	-
Cardiac glycosides	-	+

(+): Present; (-): Absent.

3.2. Quantitative Phytochemical Analysis and Percentage Yields

The contents of total phenols, flavonoids, condensed tannins and saponins were determined from the linear regression equations of each calibration curve: y=0.0161x + 0.0301 (R^2=0.9975), y=0.0243x - 0.0854 (R^2=0.9936), y=0.0004x + 0.0033 (R^2=0.9933) and y=0.002x - 0.0427 (R^2=0.9985) respectively. Table 2 summarizes the results obtained for the aqueous and hydroethanolic extracts. The aqueous extract contained 419.08±3.01 mg GAE/g DW of total phenolic compounds, 99.62±1.69 mg RE/g DW of flavonoids, 215.46±2.96 mg CE/g DW of tannins and 185.25±0.61 mg SE/g DW of saponins. Hydroethanolic extract contained 562.91±7.21 mg GAE/g DW of total phenolic compounds, 135.96±0.41 mg RE/g DW of flavonoids, 228.50±0,62 mg CE/g DW of tannins and 206.83±1.70 mg SE/g DW of saponins. We found that the contents of polyphenols, flavonoids, condensed tannins and saponins were high in these extracts. However, the amounts of phytochemicals evaluated from the hydroethanolic extract are higher than those from the aqueous extract.

Table 2. *Yields, contents of total polyphenols, flavonoids, condensed tannins and saponins in the aqueous and ethanolic extract of Tephrosia pedicellata.*

Extracts	Yield (%)	Total polyphenols (mg GAE/g DW)	Flavonoids (mg RE/g DW)	Condensed tannins (mg CE/g DW)	Saponins (mg SE/g DW)
Aqueous	14	419.08±3.01	99.62±1.69	215.46±2.96	185.25±0.61
Ethanolic	12	562.91±7.21	135.96±0.41	228.50±0,62	206.83±1.70

GAE/g DW: gallic acid equivalent per gram of dry weigh; RE/g DW: rutin equivalent per gram of dry weight; CE/g DW: catechin equivalent per gram of dry weight; SE/g DW: saponin from quillaja bark equivalent per gram of dry weight.

3.3. Effect of Tephrosia pedicellata Aqueous and Hydroethanolic Extracts on Haemonchus contortus

Overall, the anthelmintic activity of plant extracts and levamisole was concentration and time dependent. The hydroethanolic extract exhibited higher activity against *H. contortus* adult, egg hatching and larvae, compared to the aqueous extract. No worms' mortality and egg hatch inhibition were observed with PBS (negative control). LC_{50} and IC_{50} values were generally lower at the longer incubation time (Table 3). For the 12 and 24 hours' post-incubation time, hydroethanol extract (LC_{50}: 0.51 and 0.03 mg/mL respectively) showed no significant difference (p=0.454; p=1.00 respectively) as compared to levamisole (LC_{50}: 0.413 and 0.029 mg/mL respectively). The same pattern of result was observed at 24 hours' incubation with the aqueous extract. However, at 12 hours, levamisole significantly (p=0.003) exhibited *H. contortus* adult mortality. There were significant ($p < 0.001$) differences in egg hatch inhibition among plant extracts (IC_{50}: 1.22 and 0.769 mg/mL for the aqueous and hydroethanolic extract respectively) and levamisole (IC_{50}: 0.267 mg/mL). Besides, both aqueous and hydroethanol extracts exhibited 100% larval mortality respectively after 48 and 24 hours' post-treatment at 2.5 mg/mL. As presented in table 3, larval mortality was significantly ($p < 0.001$) high for levamisole (LC_{50}: 0.25 mg/mL) as compared to both aqueous (LC_{50}: 0.94 mg/mL) and hydroethanol (LC_{50}: 0.51 mg/mL) extracts at 24 hours' post-exposure. After 48 hours, the levamisole anthelmintic activity showed a highly significant (p=0.001) and significant (p=0.016) effect compared to those of aqueous and hydroethanolic extracts respectively.

Table 3. *LC_{50} and IC_{50} values of Tephrosia pedicellata aqueous and hydroethanolic extracts on Haemonchus contortus adult, larvae and egg hatching.*

Drugs	Adult LC_{50} (mg/mL)		Larval LC_{50} (mg/mL)		Egg hatching IC_{50} (mg/mL)
	12 h	24 h	24 h	48 h	48 h
AE	0.67±0.05**	0.17±0.00^{ns}	0.94±0.02***	0.29±0.02**	1.22±0.06***
HE	0.51±0.13^{ns}	0.03±0.01^{ns}	0.51±0.03***	0.22±0.02*	0.76±0.02***
Lev	0.41±0.05	0.02±0.00	0.25±0.02	0.12±0.03	0.26±0.02

AE: aqueous extract; HE: hydroethanolic extract; Lev: levamisole. Levamisole was used as positive control. Comparisons were made in column against levamisole following to *t* Student test. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$, ns: $p > 0.05$.

3.4. Effect of Tephrosia pedicellata Aqueous and Hydroethanolic Extract on Caenorhabditis elegans Mutant Strain (CB211) and Wild Type (WT)

The mortality of these two strains of *C. elegans* increased with ascending concentrations and incubation time, irrespective of plant extracts. The hydroethanolic extract showed a higher anthelmintic activity than that of the aqueous extract on the two strains used. There is a non-significant difference between the effect of the aqueous extract on strains CB211 and WT at all the recording times (p=0.963, p=0.991; 24 and 48 hours) (Table 4). Same results were observed with the hydroethanolic extract on strains CB211 and strain WT (p=0.400, p=0.976) at the same time periods.

Table 4. *Comparative values of LC_{50} (mg/mL) of aqueous and hydroethanolic extract of Tephrosia pedicellata on Caenorhabditis elegans mutant strains (CB211) and wild type (WT).*

Drugs	LC_{50} (mg/mL)			
	24 h		48 h	
	CB211	WT	CB211	WT
Aqueous extract	0.65±0.08^{ns}	0.63±0.1^{ns}	0.26±0.05^{ns}	0.25±0.02^{ns}
Ethanol extract	0.35±0.04^{ns}	0.31±0.04^{ns}	0.22±0.03^{ns}	0.21±0.03^{ns}
Levamisole	> 1.5	0.19±0.00	> 1.5	0.17±0.07

The comparisons were made in the lines, between levamisole resistant (CB211) and non-resistant (WT) strains assessed in the same hour (*t* Student test). ns: $p > 0.05$

3.5. Acute Toxicity

Hydroethanolic extract of *T. pedicellata* at all doses produced no visible changes in the behavioral patterns of the treated rats. No signs of toxicity such as decreased sensitivity to noise, convulsion, diarrhea, tremor, abdominal constrictions, sleep or coma, were observed.

3.6. Subacute Toxicity

3.6.1. Haematological and Biochemical Parameters

The effects of long term administration of the hydroethanolic extract of *T. pedicellata* on the hematological parameters of both female and male rats are illustrated in table 5. Globally, haematological composition of both male and female rats' blood showed no significant difference between treated and control groups ($p > 0.05$). Only platelets number significantly increased (p=0.01) at dose of 1000 mg/kg for females and males.

Results of the effect of the hydroethanolic extract of *T. pedicellata* on the biochemical parameters in female and male rats are shown in Table 6. There was no significant difference at all doses in females' biochemical parameters include urea, creatinine, ALAT and glucose compared to control group ($p > 0.05$). However, there was a significant increase in ASAT level (p=0.012) in the treated groups (1000 mg/kg) compared to untreated animals. The same patterns of results were obtained in treated male rats in comparison to the control animals (p=0.001).

Table 5. *Effect of the hydroethanolic extract of Tephrosia pedicellata on the hematological parameters of rats.*

Gender	Parameters	Doses			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
Females	WBC (10^9/L)	6.57±1.41	7.42±1.93 ^{ns}	8.46±0.09 ^{ns}	8.72±3.45 ^{ns}
	LYM (10^9/L)	5.24±0.79	5.35±1.52 ^{ns}	6.61±0.65 ^{ns}	4.93±2.24 ^{ns}
	MID (10^9/L)	1.105±0.24	0.66±0.15 ^{ns}	0.67±0.15 ^{ns}	1.3±0.28 ^{ns}
	GRA (10^9/L)	1.72±0.32	1.45±0.46 ^{ns}	1.19±0.57 ^{ns}	2.49±0.93 ^{ns}
	RBC (10^{12}/L)	6.94±0.67	7.77±0.08 ^{ns}	7.82±0.1 ^{ns}	7.20±0.37 ^{ns}
	HB (g/dl)	11.4±1.55	12.83±0.3 ^{ns}	13±0.56 ^{ns}	12.45±1.48 ^{ns}
	HT (%)	35.41±2.6	41.76±2.12 ^{ns}	44.01±1.23 ^{ns}	39.4±4.49 ^{ns}
	MCV (fl)	51±1.41	53.66±2.08 ^{ns}	56.5±0.7 ^{ns}	54.5±3.53 ^{ns}
	TCMH (pg)	16.4±0.7	16.5±0.36 ^{ns}	16.65±0.49 ^{ns}	17.3±1.13 ^{ns}
	CCMH (g/dl)	32.1±2.12	30.76±1.38 ^{ns}	29.65±0.49 ^{ns}	31.65±0.07 ^{ns}
	IDR (%)	24±1.55	23.03±1.97 ^{ns}	23.4±2.12 ^{ns}	23.05±1.90 ^{ns}
	PLT (10^9/L)	665.5±4.94	676±8.48^{ns}	682±9.89^{ns}	742.5±3.53***
	VPM (fl)	7.7±0.7	8.46±0.51 ^{ns}	9±0.42 ^{ns}	8.45±0.49 ^{ns}
	IDP (%)	39.45±0.77	40.3±1.12 ^{ns}	40.05±0.91 ^{ns}	39.9±0.7 ^{ns}
	P-LCR (%)	25.78±3.2	28.98±4.27 ^{ns}	32.46±1.95 ^{ns}	29.31±3.86 ^{ns}
Males	WBC (10^9/L)	9.71±1.01	6.23±2.45 ^{ns}	6.14±1.40 ^{ns}	7.94±0.08 ^{ns}
	LYM (10^9/L)	7.49±2.03	3.77±1.47 ^{ns}	4.40±0.67 ^{ns}	5.01±0.02 ^{ns}
	MID (10^9/L)	0.73±0.22	0.84±0.26 ^{ns}	0.69±0.11 ^{ns}	0.98±0.02 ^{ns}
	GRA (10^9/L)	1.49±0.79	1.62±0.7 ^{ns}	2.04±0.33 ^{ns}	1.945±0.07 ^{ns}
	RBC (10^{12}/L)	8.5±0.56	7.02±0.63 ^{ns}	8.50±1.66 ^{ns}	7.33±0.72 ^{ns}
	HB (g/dl)	13.45±0.49	11.05±0.77 ^{ns}	13.5±1.97 ^{ns}	12.35±0.63 ^{ns}
	HT (%)	43.36±0.07	35.49±3.96 ^{ns}	44.09±6.57 ^{ns}	40.96±0.47 ^{ns}
	MCV (fl)	51.5±3.53	50.5±0.70 ^{ns}	52±2.82 ^{ns}	52.5±0.70 ^{ns}
	TCMH (pg)	15.85±1.62	15.75±0.35 ^{ns}	16±0.84 ^{ns}	15.65±0.63 ^{ns}
	CCMH (g/dl)	31±1.13	31.2±1.41 ^{ns}	30.65±0.21 ^{ns}	30.05±1.06 ^{ns}
	IDR (%)	25.25±1.90	23.05±1.90 ^{ns}	23.5±0.98 ^{ns}	25.55±1.06 ^{ns}
	PLT (10^9/L)	752.5±4.94	792.5±9.19 ^{ns}	783.5±6.36 ^{ns}	780±7.07 ***
	VPM (fl)	8.15±0.35	7.8±0.84 ^{ns}	7.6±1.13 ^{ns}	8.05±0.07 ^{ns}
	IDP (%)	40.65±0.35	39.45±1.06 ^{ns}	38.8±3.53 ^{ns}	40.15±0.21 ^{ns}
	P-LCR (%)	27.58±2.27	24.63±6.69 ^{ns}	22.205±9.69 ^{ns}	27.45±2.17 ^{ns}

WBC: white blood cell count, LYM: lymphocyte count, MID: neutrophil count, GRA: granulocyte number, RBC: red blood cell count, HB: hemoglobin, HT: hematocrit, MCV: mean blood volume, TCMH: mean corpuscular hemoglobin content, CCMH: mean corpuscular hemoglobin concentration, IDR: red blood cell distribution index, PLT: platelet count, VPM: mean platelet volume, IDP: platelet distribution index, P-LCR: percentage of large platelet cells. The comparisons were made in the lines against the control group by the post hoc testing of Dunnett. ns: $p > 0.05$, ***: $p < 0.001$.

Table 6. *Effects of hydroethanolic Tephrosia pedicellata extract on the biochemical parameters of rats.*

	parameters	Control	Dose 250 mg/kg	Dose 500 mg/kg	Dose 1000 mg/kg
Females	Urea (mg/dl)	49.63±9.47	31.13±5.80 ^{ns}	36.95±4.45 ^{ns}	35.15±8.83 ^{ns}
	Creatinine (mg/dl)	0.46±0.05	0.56±0.05 ^{ns}	0.7±0.14 ^{ns}	0.6±0.14 ^{ns}
	ALAT (U/l)	167.66±8.96	156.33±3.78 ^{ns}	183±9.89 ^{ns}	181.5±9.19 ^{ns}
	ASAT (U/l)	252.66±8.32	245.33±5.13 ^{ns}	258.5±7.77^{ns}	303.5±6.36**
	Glucose (mg/dl)	80.24±9.34	84.37±3.99 ^{ns}	86.26±1.08 ^{ns}	73.27±7.29 ^{ns}
Males	Urea (mg/dl)	44.1±1.55	35.15±4.73^{ns}	36.03±4.12 ^{ns}	35.2±0.28 ^{ns}
	Creatinine (mg/dl)	0.55±0.07	0.55±0.07 ^{ns}	0.5±0.1 ^{ns}	0.55±0.07 ^{ns}
	ALAT (U/l)	169.5±3.53	175.5±7.77 ^{ns}	180.33±6.65 ^{ns}	182±4.24 ^{ns}
	ASAT (U/l)	266±5.65	267.5±6.36 ^{ns}	278.66±5.03^{ns}	306.5±3.53**
	Glucose (mg/dl)	82.03±2.92	91.05±2.74 ^{ns}	75.97±8.92 ^{ns}	88.37±1.70 ^{ns}

ALAT: analine aminotransferase, ASAT: aspartate aminotransferase. The comparisons were made in the lines against the control group by the post hoc testing of Dunnett. **: $p < 0.01$, ns: $p > 0.05$.

3.6.2. Histopathological Evaluation

Figure 1 (a and b) shows the histopathological effects of hydroethanolic extract of *T. pedicellata* on the liver, kidneys and lungs tissue of females and males respectively, in both untreated (G1) and treated animals (G2, G3, G4; respectively with 250, 500 and 1000 mg/kg of plant extract). These findings showed no alteration of both liver and kidney tissues of rats treated with all plant extract' doses as compared to the control animals. However, in females and males (1000 mg/kg), some histopathological abnormalities in lung tissue, namely inflammatory granuloma were observed.

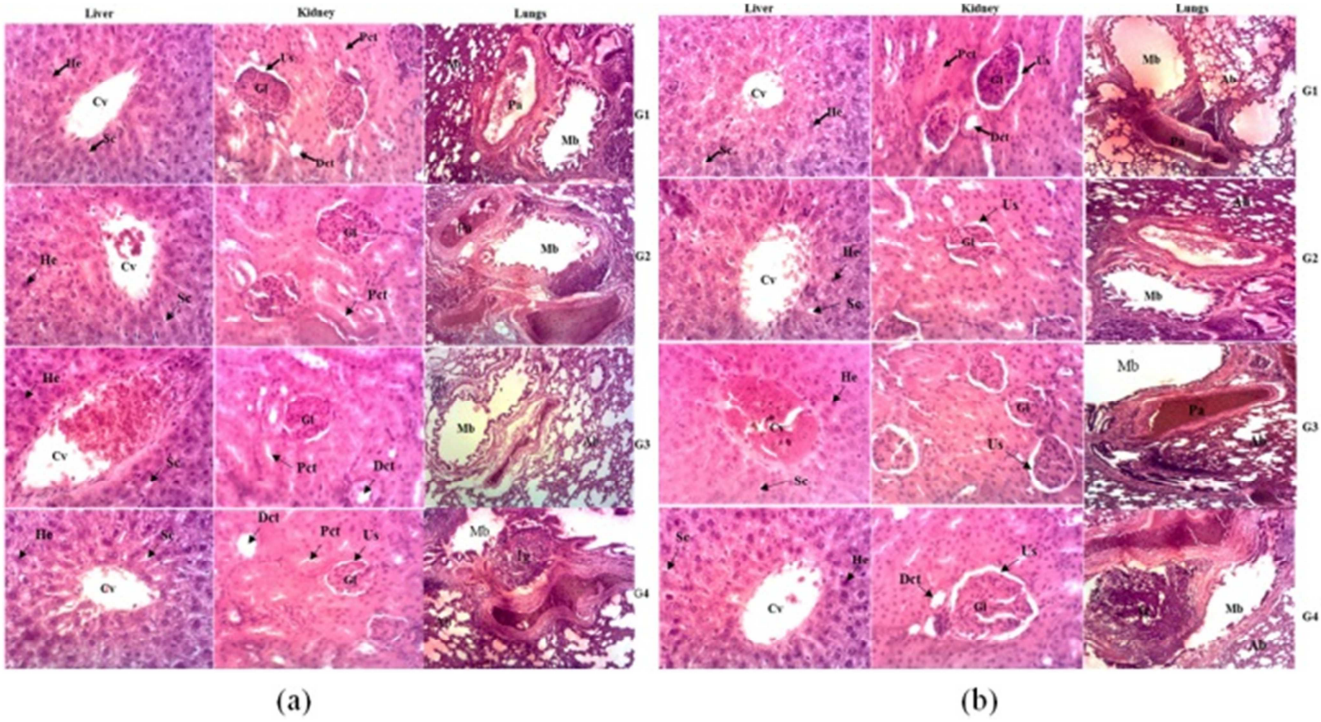

Figure 1. *Photomicrographs of the liver (X200), kidney (X200) and lungs (X40) of female (a) and males (b) rats.*

Liver: Cv=central vein; He=Hepatocyte; Sc=Sinusoidal capillary; Kidney: Gl=Glomerulus; Us=Urinary space; Dct=Distal convoluted tubule; Pct=Proximal convoluted tubule; Lungs: Mb=Main bronchus; Pa=Pulmonary arteriole; Ab=Alveolar bag; Ig=Inflammatory granuloma. G1: group 1; G2: group 2; G3: group 3; G4: group 4.

4. Discussion

Several methods have been developed *in vivo* or *in vitro* to study the anthelmintic activity against nematodes [36]. *In vitro* studies are used to quickly assess the potential anthelmintic activity of various plant extracts and to analyze possible mechanisms involved in the interaction between the active compounds and the parasites [37]. The results showed that the aqueous and hydroethanolic extracts of *T. pedicellata* are very effective on the three developmental stages of *H. contortus* and on the free nematode *C. elegans*. The results obtained in this study are in line with those reported from other plant extracts with promising anthelmintic activity on *H. contortus* and *C. elegans* such as *Allium sativum* [38], *Khaya senegalensis* [39], *Parkia biglobosa* and *Pterocarpus*

erinaceus [27]. The hydroethanolic extract of this plant showed higher anthelmintic activity than the aqueous extracts. Phenolic compounds such as tannins and flavonoids have been widely shown to have anthelmintic activity [40, 14]. The phytochemical study carried out showed that the hydroethanolic extract contained more phenolic compounds and saponins than the aqueous extract. Along the same lines, some authors [41, 42] showed that ethanol associated with water allows better extraction of phenolic compounds than water alone. This could explain why the hydroethanolic extract was more effective than the aqueous extract in this study.

To our knowledge, *T. pedicellata* has never been tested against *H. contortus* and *C. elegans*. In this study, the anthelmintic activity of *T. pedicellata* could be attributed to one or more phytochemicals contained in the extracts of this plant such as polyphenols, flavonoids, tannins, saponins, alkaloids, terpenoids and cardiac glycosides. Some of these observed phytochemicals (phenolics and saponins) have been demonstrated by Castañeda-Ramírez *et al*. [14] as having anthelmintic activity. The assays carried out with the aqueous and hydroethanolic extract of *T. pedicellata* clearly show the presence of phenolic compounds, condensed tannins, flavonoids and saponins at high levels. The strong anthelmintic activity demonstrated by this plant against the parasitic nematode *H. contortus* and the free nematode *C. elegans* resistant to levamisole and wild type could be related to the presence of these compounds.

The larvicidal activity and adult mortality observed in our results may be due to the penetration of active phytochemicals through the cuticle of larvae and adult worms [43]. According to [44], the effect of tannins is similar to that of certain synthetic phenolic anthelmintics such as niclosamide and nitroxynil, which interfere with energy production by decoupling oxidative phosphorylation in parasitic helminths. Tannins also have the potential to directly inhibit oxidative phosphorylation which causes starvation and ultimately worm death [45]. Regarding the inhibition of egg hatching, tannins or saponins eventually penetrated the different membranes of the egg and inhibited larval formation by affecting the morula [14]. Several studies have shown that saponins and tannins can stop larval formation and the hatching process of *H. contortus* eggs [46, 47]. In addition to their ability to stop larval formation, these phytochemicals can also inhibit the hatching of eggs containing a larva [48]. According to [49], saponins are known to destabilize cell membranes and therefore increase cell permeability by combining with the membranes of associated sterols. These molecules are also known to prevent the hatching of *H. contortus* eggs [50]. According to [37] flavonoids also have effective anthelmintic properties. Brunet [51] reported that the larval sheath is the target of the flavonoids contained in sainfoin.

Levamisole is a cholinergic agonist ionotropic which selectively produces a depolarization of muscle cells of nematodes and spastic contraction [52]. It thus causes muscle hypercontraction and mortality due to the prolonged activation of excitatory nicotinic acetylcholine receptors on muscles. Indeed, three genes *unc*-38, *unc*-29 and *lev*-1 encode subunits of the non-alpha-nicotinic acetylcholine receptor which confer resistance to levamisole when mutated [53]. The *lev*-1 gene allows normal locomotion and forms a cation channel when co-expressed with *unc*-38 or *unc*-63 and *unc*-29 and is expressed in the muscle [53]. Since the anthelmintic activity of aqueous and hydroethanolic extracts of *T. pedicellata* on levamisole resistant strains CB211 is similar to the effect on wild type of *C. elegans*, it is therefore likely that *lev*-1 is not the binding site of the molecules of the extract and that the plant probably does not act on the muscles but elsewhere.

The study of the toxicity of a therapeutic substance used in traditional medicine is necessary to determine its safety with a view to its use without risk of intoxication [54]. Data obtained from an animal acute oral toxicity test can be used to meet hazard classification needs through the LD_{50}, and for the assessment of risks to human, animal and / or environment [33]. It is on this basis that an evaluation of the acute oral toxicity in rats of the extract of *T. pedicellata* was carried out. No mortality was obtained at all doses. This value of LD_{50} made it possible to classify the toxicity of the hydroethanolic extract of *T. pedicellata* in category 5 of the globally harmonized classification system for chemical substances, a category characterizing substance of low toxicity [33]. No treatment-related changes were observed over the remainder of the observation period, nor any decrease in weight gain. These results therefore suggest that this extract presents very low health risks in a single administration. To our knowledge, no toxicity studies have been performed with *T. pedicellata*.

Repeated dose toxicity studies are conducted to evaluate the adverse effects of a test substance after prolonged use. They are carried out to provide information about the possible health hazards likely to arise from repeated exposure over a relatively limited period of time including information about target organs, the possibilities of cumulative effects, and an estimate of the dose at which there is no observed adverse effect [55]. Oral administration of hydroethanolic extract of *T. pedicellata* for 28 days does not alter all haematological parameters compared to the control. However, this extract caused a very significant increase (p=0.001) in blood platelets compared to control at the dose of 1000 mg/mL. Blood platelets are one of the main elements of hemostasis but are also involved in the etiology of cardiovascular pathologies [56]. The physiological response to a breakdown in the integrity of a vascular endothelium consists in particular of a localized and rapid activation and aggregation of platelets [57]. These will make it possible to plug the vascular breach, a reaction which may be sufficient in the case of low shear forces. Monitoring platelet function is important. Indeed, platelet hyperactivity can cause thrombosis which can cause complications such as pulmonary embolism, heart attack and cerebrovascular accident [56].

Kidneys and liver being the preferred targets of toxic substances, once damaged, these organs release their enzymatic or protein contents into the blood. Their assessments in serum are useful markers for the extent and type of liver or kidney damage [58]. Transaminases (ALAT

and ASAT) are enzymes with significant metabolic activity inside cells. These are transferases that are involved in chemical reactions in the body and lead to the creation of new amino acids, necessary for the body to function properly. ALAT is found in the liver specifically and ASAT is found abundantly in heart muscles and other organs such as the lungs. The results show that the ALAT values are not modified in animals treated with hydroethanolic extract of *T. pedicellata* by intake in control and at all doses. Only ASAT values increased at the 1000 mg/kg dose. The increase in serum ASAT may therefore reflect cellular damage, especially in the heart and lungs [59]. These results could explain the breathing difficulties in animals treated with large doses of the hydroethanolic extract of *T. pedicellata*. These results are similar to those obtained in a similar study by Unuofin *et al*. [60] with aqueous extract of *Kedrostis africana*, which observed a significant increase in ASAT. Renal function was assessed by serum urea and creatinine assays. The kidney workup is used to screen for possible kidney dysfunction. Indeed, these parameters have high values in case of alteration of the renal filtration mechanism [61, 62]. Serum urea and creatinine are considered to be the primary markers of nephrotoxicity, although serum urea is often considered a more reliable predictor of renal function than serum creatinine [63]. Hydroethanolic extract of *T. pedicellata* did not alter renal structure and function at administered doses.

Photomicrograph of the liver and kidneys of animals treated with the hydroethanolic extract of *T. pedicellata* did not show any abnormalities. However, the lungs showed the presence of an inflammatory granuloma at a dose of 1000 mg/kg. The term granuloma refers to any nodular organized aggregation of mononuclear inflammatory cells or collection of modified macrophages, usually surrounded by a rim of lymphocytes and often containing multinucleated giant cells. Some granulomas may also contain eosinophils and plasma cells [64]. This inflammation could be as a result of the damage caused by the extract of *T. pedicellata* and could explain the high level of ASAT observed at the highest dose.

5. Conclusion

The present study shows that aqueous and hydroethanolic extracts of *T. pedicellata* have evidence *in vitro* anthelmintic activities against three-stages of *H. contortus* and *C. elegans* adult. The high amount of phenolic compounds and saponins contained in this plant could be responsible to its anthelmintic activity. The limit dose of 2000 mg/kg does not show any toxic effects on rats. Daily intake for 28 days of 250 and 500 mg/kg of plant extracts did not affect the haematological and biochemical parameters of rats, while, at a dose of 1000 mg/kg caused an increase in the quantity of platelets and AST and caused glomerular inflammation. From these observations, it can be assumed that the leaves of *T. pedicellata* are good source of traditional medicines against helminthic infections. Further studies will be aim to isolate and purify potential compounds from this plant extract. Also, further studies considering the metabolism of the extract in the digestive tract of the animal should be done by evaluating its *in vivo* activity.

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