Trypanocidal activity of aqueous and ethanolic extracts of *Leptadenia hastata* on white mice infected with *Trypanosoma congolense*

ABSTRACT

Aims: The aims of this study is to evaluate the trypanocidal activity of the plant extracts on mice infected with the trypanosome strain.

Study Design: The retrospective study was conducted from June to November 2020 at the laboratory of animal Biology, Department of Biological Sciences, University of Ngaoundéré, Cameroon.

Methodology: The trypanocidal activity of *Leptadenia hastata* aqueous and ethanolic extracts on *Trypanosoma congolense* was evaluated during ten days of treatment. Thus, the smear was used to monitor the parasites density and its mortality rate in mice treated with *Leptadenia hastata* aqueous and ethanolic extracts by gavage at doses of 250, 500, 1000 mg/kg (test groups); with 10 ml/kg of distilled water (negative control) and 1mg/kg of isometamidium chloride (positive control). The lethal dose 50 (LD_{50}) was evaluated using the equation of the linear regression line obtained with the mortality probits at day 10 of treatment.

Results: Phytochemical analysis showed the presence of steroid, Alkaloids, Saponosides, Flavonoids, tannins and triterpene. The 1000 mg/kg dose of the aqueous extract on day six (D_6) right up to day eight (D_8), induced a statistically decrease in parasites density which is similar to that of isometamiduim chloride. At day 0 (D_0) the parasite density was 500 parasites/µl and at day 10 (D_{10}) it was reduced to 250 parasites/µl with the 1000 mg/kg dose of the ethanolic extract. Parasite mortality was induced by 35 % by the 250 mg/kg dose, 60 % by the 500 mg/kg dose and 41 % by the 1000 mg/kg dose of the aqueous extract on day ten (D_{10}) of treatment. With the ethanolic extract, parasite mortality on day 10 day of treatment was induced by 48 % by the 250 mg/kg dose, 68.75 % by the 500 mg/kg dose and 59.18 % by the 1000 mg/kg dose. Isometamidium chloride, a positive control, induced a parasite mortality rate of 85 %. The LD_{50} was 229.07 mg/kg for the aqueous extract and 271.37 mg/kg for the ethanolic extract.

Conclusion: All these results justify at least in part the use of this plant in traditional medicine for the treatment of trypanosomiasis.

Keywords: Ethanolic extracts; Aqueous extracts; Trypanocidal potential; Trypanosomes; Inhibition of parasitaemia.

Abbreviation: HAT: Human African Trypanosomiasis, AAT: African Animal Trypanosomiasis, $DL_{50:}$ Lethal dose 50, WHO: World Health Organization, ANOVA: Analysis of Variance, T: Mortality rates, TN: Negative Control, TP: Positive Control, W: Weignt, V: Volume, mg: milligram, kg: kilogram.

INTRODUCTION

Trypanosomoses are parasitic diseases caused by protozoa belonging to the genus *Trypanosoma* and the family Trypanomastidae, which multiply in blood plasma, lymph and various tissues, including the heart muscle and central nervous system of mammals [1]. Tsetse flies are the pathogens of both HAT and AAT [2]. They therefore play a key role in the epidemiology of trypanosomiasis through their central role in the transmission of trypanosomes to vertebrate hosts [3].

The development of cattle breeding has been consistent with the control of this disease. Despite these available results, trypanocides remain the most widely sold drugs [4]. Currently available treatments (Suramin and pentamidine in the blood stage, melarsoprol and eflornithine in the advanced stage) are outdated, some of them even proving to be toxic to patients [5]. Several approaches to control the vector, such as breeding trypan tolerant animals, using biotopes unfavorable to vector development and using trypanocides are used [6]. Vector control using insecticides of varying degrees of persistence are also used, but these are involved in the destruction of the useful insects such as bees, which play an important role in pollination and honey production. The limitations of these different control methods and the appearance of trypan-resistance have been demonstrated worldwide [7]. The chemo resistance of trypanosomes to the current drugs used since 1940 is becoming an alarming problem, due to the lack of therapeutic alternatives that seriously compromise the control of this parasite [8]. The need for new, less expensive and non-toxic trypanocides has been pressing for almost a century. It is also known that locally used plants are important source of new drugs [9]. Thus, in view of the poverty afflicting African countries, the evaluation of the trypanocidal activity of medicinal plants remains an important area of research.

Leptadenia hastata is a typical forest and fruit species. It is a thorny shrub with a beautiful appearance that reminds one of the jujube tree in terms of its foliage and thorns. *Leptadenia hastata* is a well-known strong medicinal plant. It is presented as an anti-venomous serum, very good antispasmodic, anti-diarrheal and excellent purgative. It is also used as an anti-malarial, galactogen, against nerve problems and dermatosis [10].

Medicinally, *Leptadenia hastata* has many applications. The latex is applied to wounds and inserted into the nose for headaches. Decoctions and macerations of roots and leaves are applied (alone or in combination with preparations of other plants) against abdominal pain such as constipation, urethral discharge, gonorrhoea, stomachache and diarrhoea. In veterinary medicine, the plant is used against colic in horses and cattle [11].

This study aims to validate the trypanocidal traditional use of *Leptadenia hastata*, the activity of the aqueous and ethanolic extracts was evaluated on mice infested with *Trypanosoma congolense*.

1. MATERIALS AND METHODS

1.1. Plant material

The ethnobotanical survey was carried out among the traditional healers in Chad, (Africa), and the recipes and plants most commonly used for the treatment of the disease were selected for the experimental studies in the laboratory. Thus, the mature leaves of *Leptadenia hastata* were chosen. The mature leaves of *Leptadenia hastata* were collected the months of June 2020, from Léré (southwestern Chad, Africa) a locality located 35 km from Figuil (North Cameroon, Africa). The Identification was carried out at the National Herbarium of Yaoundé/Cameroon where the voucher was kept under specimen number: 40786/SRF. The leaves were washed with water

and then dried under artificial ventilation, free from direct sunlight and dust. Once dried, the leaves were grind into powder and sealed in airtight bags.

Figure 1: *Leptadenia hastata* [12]

1.2. Animal material

The study was carried out on white mice *Mus musculus* Swiss, of both sexes weighing between 20 and 32 g. They were supplied by the National Veterinary Laboratory (LANAVET of Garoua (Cameroon), and then acclimatized for 7 days in the Animal Physiology Laboratory of the University of Ngaoundéré. The animals were fed with pellets supplied by LANAVET and *adlibitum* water.

Figure 2: *Mus musculus Swiss* mouse

1.3. Trypanosomal strain

The *Trypanosoma congolense* strain used in this study to infect mice are originated from the Faculty of Veterinary Medicine, University of Nigeria Nsukka.

1.4. Chemical substance

Isomethamidium chloride (Trypamidium®) which was used as a reference substance in this study was purchased from Santa Cruz Biotechnology (CAS 6798-24-9). It is in the form of a red powder, which is soluble in water.

1.5. Preparation of extracts

One hundred and fifty grams of the crushed material was macerated in 500 ml of distilled water. The maceration was carried out for 24 hours under magnetic stirring, protected from light by covering the beakers with aluminum foil. The macerate was then collected and centrifuged. The supernatant was filtered, frozen and freeze-dried. After 72 hours of freeze-drying, a powder was obtained with a percentage yield of 24.6%. The lyophilisate was kept in a desiccator to avoid any humidification.

$$\text{Percentage yield} = \frac{\text{Mass of power extract}}{\text{Mass of concentrated filtrate}} \text{ X } 100$$

1.5.1. Preparation of ethanolic extracts of *Leptadenia hastata*

One gram of lyophilisate was taken and dissolved into 10 ml of 70 % ethanol. The stock solution of concentration 100 mg/ml was obtained and corresponded to the dose of 1000 mg/kg (D_1). This solution was dissolved into ½ and ¼ in distilled water and yielded the respective doses of 500 (D_2) and 250 mg/kg (D_3).

1.5.2. Preparation of aqueous extracts of *Leptadenia hastata*

One gram of dry extract of *Leptadenia hastata* was taken and dissolved into 10 ml of distilled water. The stock solution of concentration 100 mg/ml was obtained and corresponded to the dose of 1000 mg/kg (D_1). This solution was dissolved into ½ and ¼ in distilled water, and yielded the respective doses 500 (D_2) and 250 mg/kg (D_3).

1.6. Qualitative analysis of phytochemical constituents

The qualitative phytochemical screening was realized in order to determine the presence of tannins, saponosides, triterpenes, flavonoids and alkaloids in the aqueous and ethanolic extracts of *Leptadenia hastate* leaves.

1.6.1. Determination of tannins

Two drops of 2 % $FeCl_3$ solution was added to 2 ml of the test solution and the mixture was left to stand for 5 minutes. A positive test was revealed by the appearance of a blue-black coloration and a precipitate, which confirms the presence of tannins [13].

1.6.2. Determination of saponosides

Five millimeters of three solvents: etheric, ethanolic and aqueous were thoroughly mixed with 10 ml of distilled water for 2 minutes. The mixture was shaken vigorously, and the formation of a persistent foam after 15 minutes confirmed the presence of saponosides [13].

1.6.3. Determination of triterpenes

Five millimeters of each extract was mixed with 2 ml of chloroform and 3 ml of concentrated sulphuric acid. A brownish-red color of the interface layer indicated the presence of heterosidic triterpenes [14].

1.6.4. Determination of flavonoid

Five millimeters of *Leptadenia hastata* extracts were mixed with a few drops of concentrated HCl and a quantity of magnesium turnings was added (leaving to act). The presence of aglycone flavones was confirmed by the appearance of a red or orange color [13].

1.6.5. Determination of alkaloid

A few drops of Mayer's reagent were added to 1 ml of *Leptadenia hastata* extracts. The formation of a white precipitate indicated that the test was positive.

1.7. Distribution and infestation of mice

Eighty-four mice of about 8 to 10 weeks' old were selected homogeneously according to their weight (20 to 32 g). These mice marked on their tails were distributed into 14 groups of 6 mice each in polystyrene cages: 1 negative control group, 1 positive control group and 12

test groups. After one week of acclimatization in the laboratory, the mice were inoculated by an intraperitoneal injection of about 0.05 ml of blood containing about 1000 strains of *Trypanosoma congolense* trypanosomes. After injection, the animals were placed in cages with the same environmental conditions as before. Parasitemia was checked on day 4 post-infection, and then the mice were treated on day 5 with the different doses of the extracts (250, 500 and 1000 mg/kg) and isometmidium chloride (1mg/kg). As from the 7th day, a cut was made on the terminal part of the tail of each animal every 2 days during 10 days. After the cutting of the tail, a drop of blood was deposited between slide and coverslip for the observation under the microscope and the number of parasites per field was recorded. The evolution of mice body weight was also evaluated. The pronunciation of clinical symptoms (coat, behavior, noticeable emaciation) and mortality during the infection were recorded.

1.8. Treatment of infected mice

The mice divided as described above were treated with different treatments. The negative control group was treated with distilled water (by oral pathway), the positive control group was received a dose of 1mg/kg of isometamidium while the 12 test groups were treated orally with different doses (250, 500 and 1000 mg/kg) of the aqueous and ethanolic extracts. After treatment, mice were monitored for 10 days and fresh blood was drawn (50 µl) by cutting off the terminal part of the tail of each mouse every 2 days for parasitaemia analysis.

1.9. Realization of blood smears

A drop of blood from the tip of the tail of each mouse was placed on a slide. Another slide was used to spread the drop. A smear was prepared following the usual hematology procedure. The smear was air-dried and then fixed with methanol for one minute. Excess methanol was removed by turning the smear downwards onto a staining tray. Using a 20 ml syringe and a blunt-tipped needle, the Giemsa was diluted 1:10 with buffered distilled water. After mixing, the Giemsa was removed by air. Using the needle and syringe, the Giemsa solution was introduced under the slide, taking care to avoid trapping large air bubbles.

The whole set was left for 30 minutes. At the end of the staining time, the slides are rinsed briefly with running water and then left to dry in an upright position. Any parasites were observed with an immersion objective for further detail of their morphology. Immersion objectives x 100 were particularly useful in the preliminary examination. Several microscopic

fields are scanned to determine the presence or absence of trypanosomes.

1.10. Statistical analysis:

Blood parasite densities (D) were determined using the following formula from [15]:

$$D = \frac{\text{Number of parasites}}{\text{Number of fields read}} \text{ X } 50$$

The number of parasites corresponds to the number of trypanosomes in 100 slide fields. Mortality rates (T) are determined by the following formula from [16]:

$$T = \frac{(A-B)}{A} \text{ X } 100$$

A and B are the parasite densities before and after treatment respectively, the same animal being considered as a control before treatment. The difference between the positive control and treated groups was statistically analyzed by the analysis of variance (ANOVA) method followed by Dennett's multiple comparison tests, using Graph Pad Instate software. P-values of less than 5% ($P<0.05$) are considered statistically significant. The lethal doses 50 (LD_{50}) were calculated using the equation of the linear regression line expressed as follows $Y= A x +B$

x is the value of the decimal logarithm of the doses, assuming that for LD50, Y= 5, then x = (5-B)/A.

2. RESULTS

2.1. Phytochemical characterization tests

Table 1: Phytochemical composition of *Leptadenia hastata*

Families of chemical compounds	Decoction
Alkaloids	+
Saponosides	+
Flavonoids	+
Tannins	-
Triterpenes	-

Legends: +: Present; -: Absent

2.2. Overall parasite densities before treatment

Observation of parasite densities before treatment ranged from 250 to 800 parasites/µl (Figure 3). We found out that, the 84 mice inoculated with the *Trypanosoma congolense* strain, where all parasitic. More than 70 % of the mice showed a high parasite density [400-650]. Mice with low parasite densities ranged from [250-350] and those with very low parasite densities were less numerous [700-800].

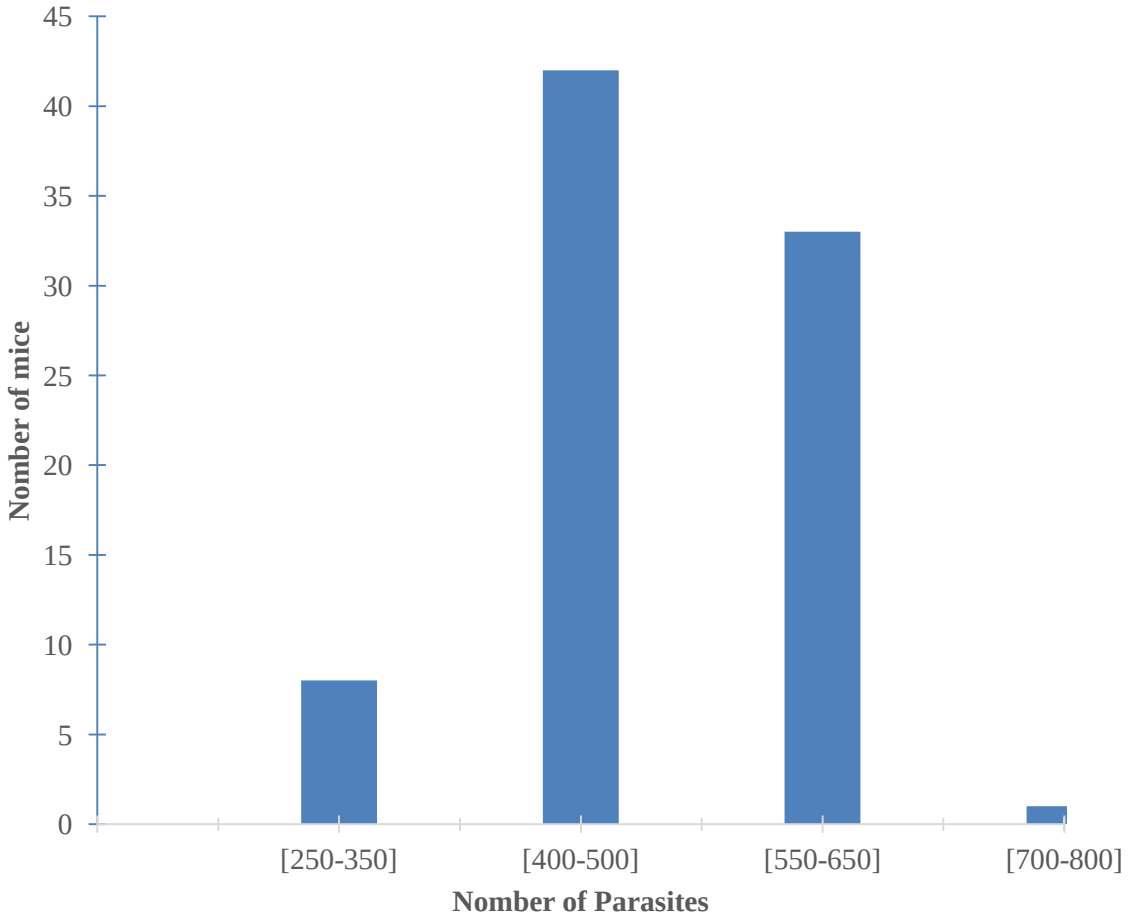

Figure 3: Overall parasite densities from 0 to 4 days before treatment with plant extracts and isometamidium chloride.

2.3. Effects of *Leptadenia hastata* on parasite numbers

2.3.1. Effect of aqueous extract on parasite density

In each batch, the different treatments resulted in a progressive decrease in parasite densities as the time after treatment increased. During the ten days (D_{10}) after treatment, the 250 and 500 mg/kg dose had a statistically similar trypanocidal effect. The 1000 mg/kg dose on day six (D_6) right up to day eight (D_8), induced a statistically decrease in density which is similar to that of isometamiduim chloride (Figure 4). On day ten (D_{10}), there was a significant difference between the 1000 mg/kg dose and isometamiduim chloride.

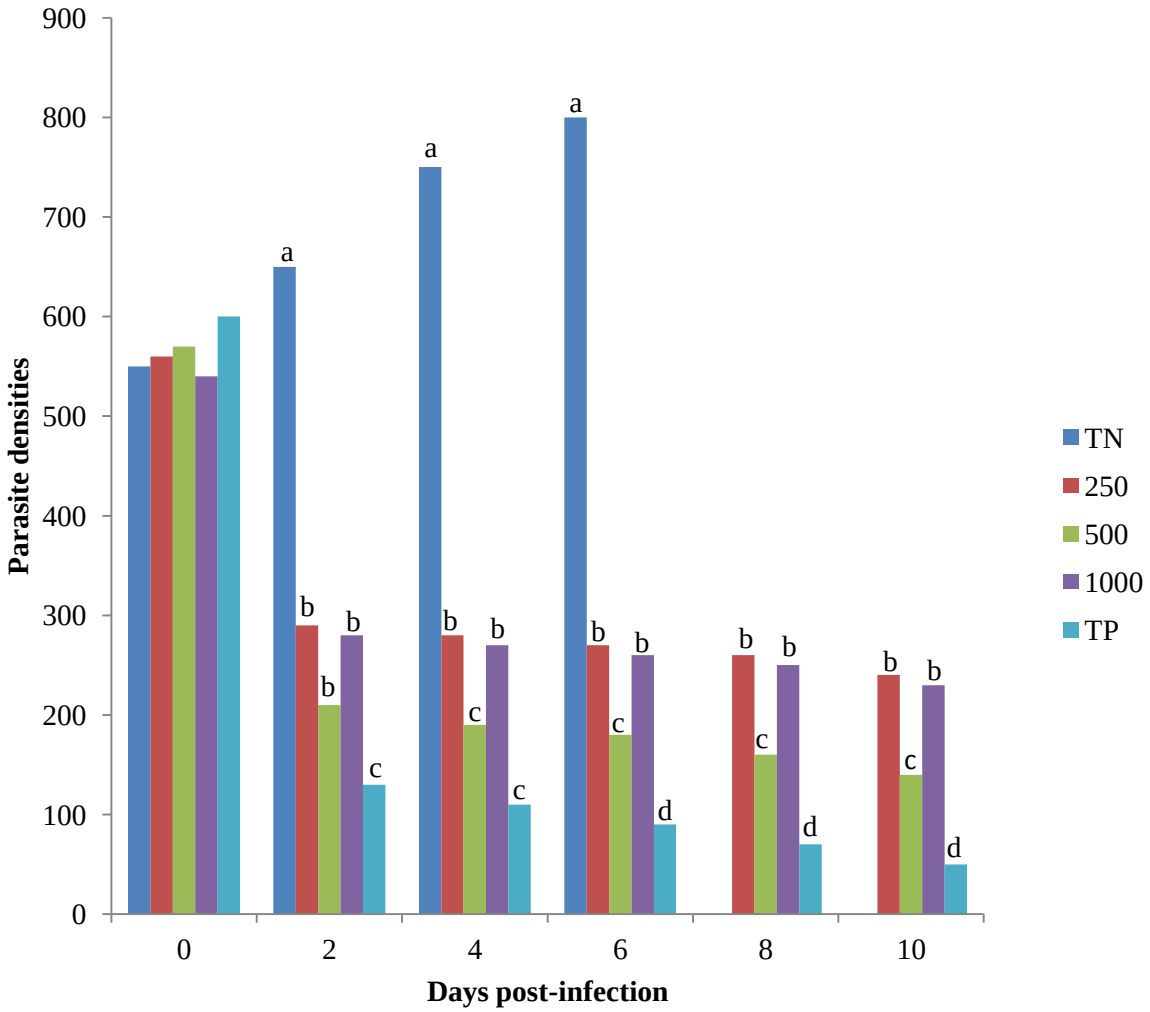

Figure 4: Effect of aqueous extract of *Leptadenia hastata* on the daily evolution of *Trypanosoma congolense* numbers

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2.3.2. Variations in parasite mortality rate in relation to the effect of the aqueous extract

The different results showed that the high dose (1000 mg/kg) has a potential effect compared to the low doses (250 and 500 mg/kg). On day ten (D_{10}) of treatment, parasite mortality was induced by 35 % by the 250 mg/kg dose, 60 % by the 500 mg/kg dose and 41 % by the 1000 mg/kg dose of the aqueous extract of *Leptadenia hastata* in mice infested with *Trypanosoma congolense* (Figure 5). On the other hand, isometamidium chloride (1mg/kg), a positive control, induced a mortality rate of 85 %.

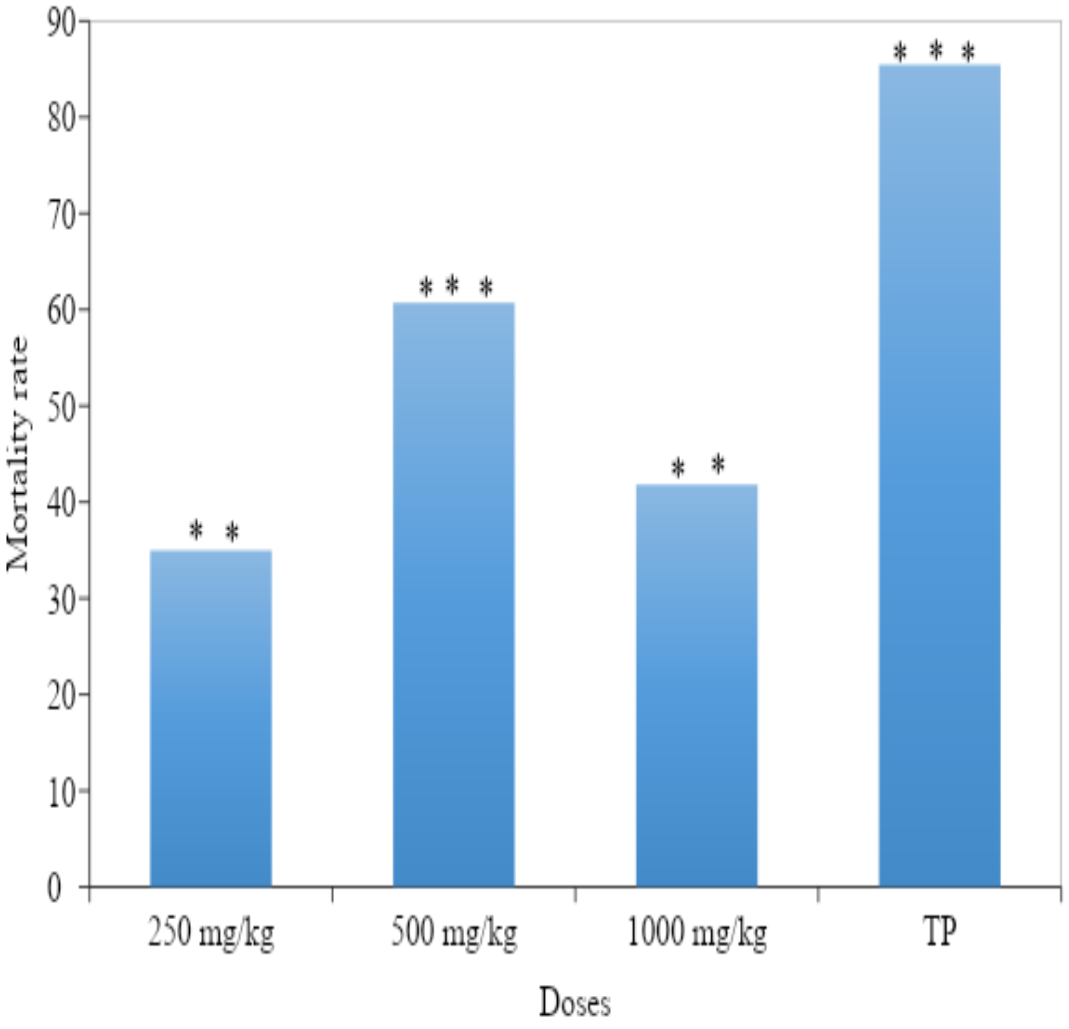

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Figure 5: Mortality rate of the effect of the aqueous extract of *Leptadenia hastata* on the parasite density of *Trypanosoma congolense*

2.3.3. Lethal Dose 50 (LD_{50}) of the aqueous extract of *Leptadenia hastata*

The equation of the linear regression line obtained with the mortality probits at day 10

of treatment is y= 0.307x + 2.209 (Figure 6).

The LD_{50} of the aqueous extract of *Leptadenia hastata* is 229.07 mg/kg on day 10.

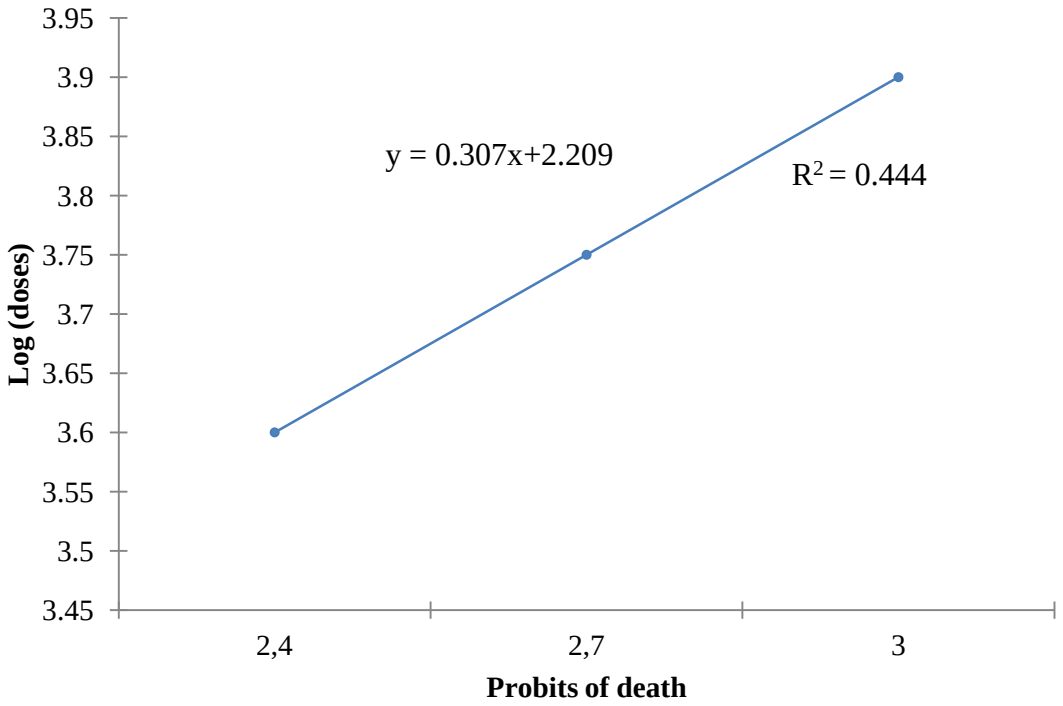

Figure 6: Regression line of *Trypanosoma congolense* mortality versus the decimal logarithm of the doses of the aqueous extract of *Leptadenia hastata*.

2.3.4. Effect of ethanolic extract on parasite density

The effect of the *Leptadenia hastata* extract was a function of the treatment days and the doses administered. The decrease was progressive over time. The 1000 mg/kg dose induced a significant decrease compared to the doses (250 and 500 mg/kg) (Figure 7). At day 0 (D_0) the parasite density was 500 parasites/µl and at day 10 (D_{10}) it was reduced to 250 parasites/µl in the 1000 mg/kg treated group. Until day 6, the effect of isometamidium chloride was comparable to that of the 1000 mg/kg dose (Figure 7). The 250 and 500 mg/kg doses showed a statistically similar trypanocidal effect from day 2 until day 10. The 1000 mg/kg dose and isometamidium chloride have a statistically similar effect on day 2 until day 3 and then its effect differs significantly on day 6 until day 10.

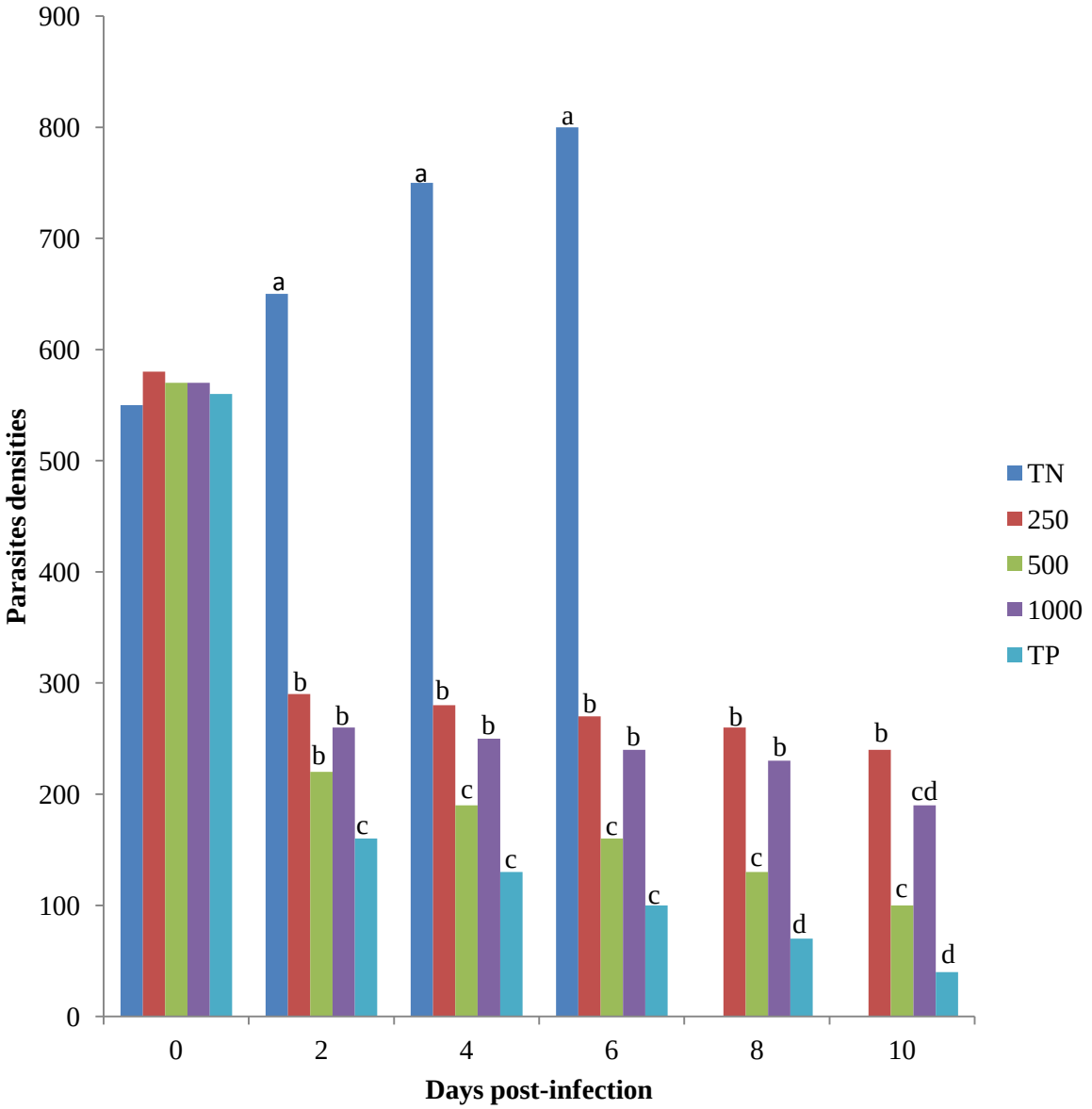

Figure 7: Effect of ethanolic extract of *Leptadenia hastata* on the daily evolution of *Trypanosoma congolense* numbers

2.3.5. Variations in parasite mortality rate by the effect of ethanolic extract

On the tenth day (D_{10}) of treatment, all doses induced a mortality rate higher than 50 %. Parasite mortality on the tenth day of treatment was induced by 48 % by the 250 mg/kg dose, 68.75 % by the 500 mg/kg dose and 59.18 % by the 1000 mg/kg dose. Isometamidium chloride, a positive control, induced a mortality of 85 %

(Figure 8).

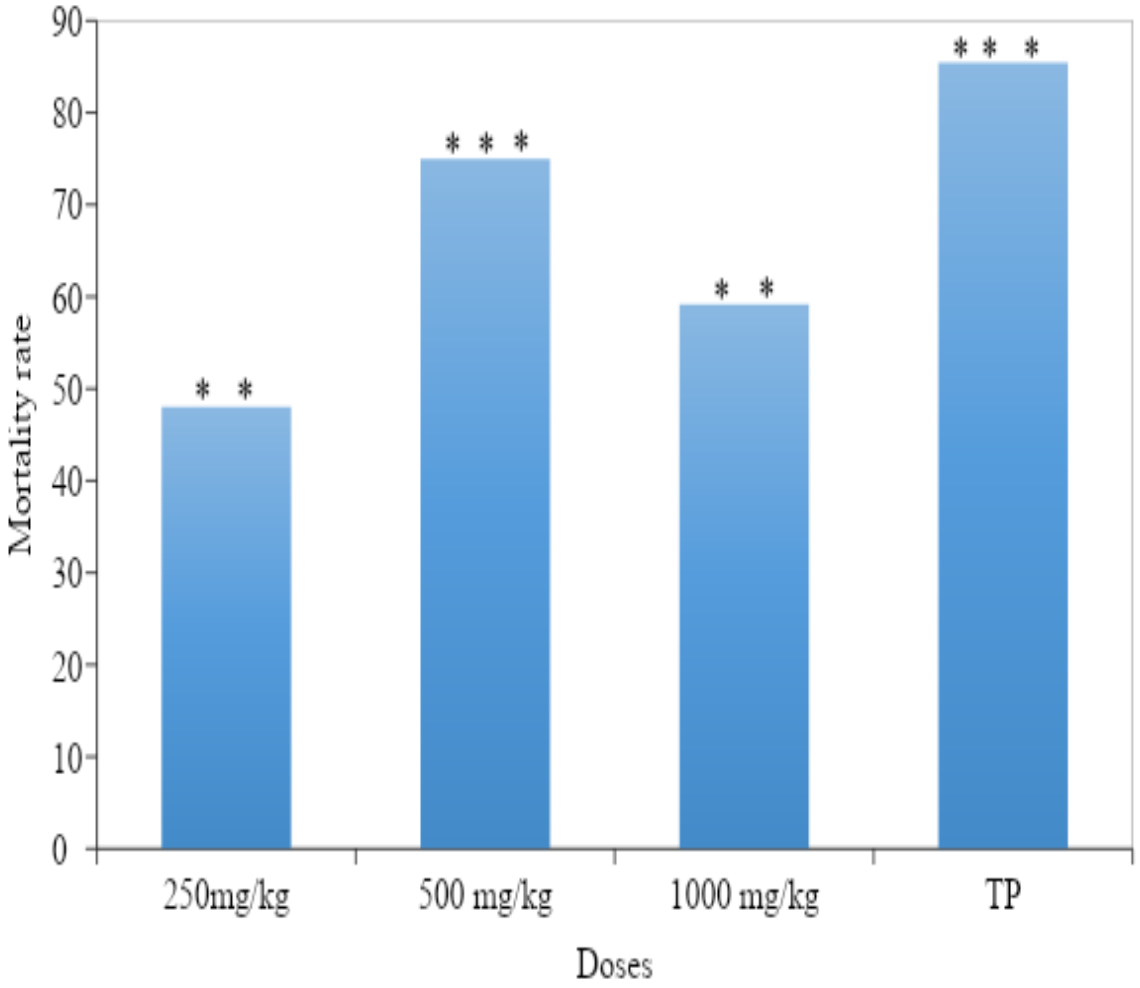

Figure 8: Mortality rate of the effect of ethanolic extract of *Leptadenia hastata* on the parasite density of *Trypanosoma congolense*

2.3.6. Lethal dose 50 (LD_{50}) of ethanolic extract *of Leptadenia hastata*

The equation of the linear regression line obtained with the mortality probits at day 10 of treatment is y= 0.322x + 2.193 (Figure 9).

The LD_{50} of the ethanolic extract of *Leptadenia hastata* is 271.37 mg/kg on day 10.

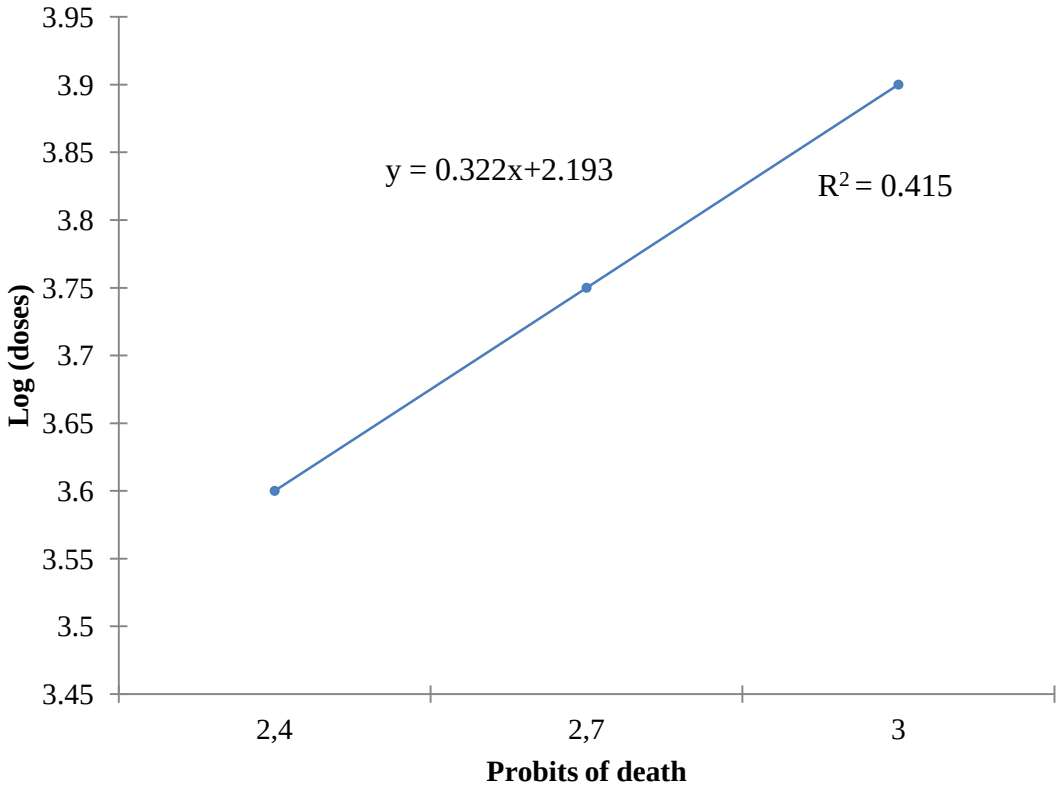

Figure 9: Regression line of *Trypanosoma congolense* mortality versus the decimal logarithm of the doses of ethanolic extract of *Leptadenia hastata*.

DISCUSSION

More than 80 % of the population in developing countries use medicinal plants for their health problems. Despite this extensive use, very little scientific work is undertaken to verify the safety and efficacy of remedies derived from these plants [17]. In this study, ethanolic and aqueous extracts of *Leptadenia hastata* foliage were tested on mice infected with *Tryapanosoma congolense* to demonstrate competition between different doses of isomethamidium chloride and the doses of 250 and 500 mg/kg of *Leptadenia hastata*. Between these doses, aqueous and ethanolic extracts of *Leptadenia hastata* showed trypanocidal activity.

The results of the overall parasite densities before treatment of the 84 mice inoculated with the *Trypanosoma congolense* strain showed that more than 70 % of the mice showed high parasite density. White mice are very sensitive hosts to trypanosomes in general and *Trypanosoma congolense* in particular. Several authors have used this parasite in white mice. This is the case of Matovu et al [18] who showed the role of IgM and B-cells antibodies in *Trypanosoma congolense* infected mice and Balé Bayala et al [19].

The effect of the aqueous extract treatments on the parasite density of mice in each batch resulted in a progressive decrease in parasite densities as the time after treatment increased. Ten days after treatment, the 250 and 500 mg/kg doses had a statistically similar trypanocidal effect. These results are comparable to those obtained by several authors [6, 20, 19] on the competitive effect of aqueous extracts of the plant and testosterone propionate on impuberate-castrated mice. They obtained a similar effect of two concentrations; this can be explained by the proximity of the doses which induce a similar effect (250 and 500 mg/kg) compared to the 1000 mg/kg dose. These authors show that apart from the low toxicity for the plant, the 1000 mg/kg dose is the most effective.

The different results of the variation of the parasite mortality rate in relation to the effect of the aqueous extract showed that the higher dose (1000 mg/kg) has a potential effect compared to the lower doses (250 and 500 mg/kg). On the tenth day of treatment, the increase in mortality rate was relative to the dose used. Thus, the 250 and 500 mg/kg doses had a mortality rate of less than 50 %. In contrast, the 1000 mg/kg dose and the positive control

isometamidium chloride induced a mortality of 65 % and 85 % respectively. The lethal dose 50 of the aqueous extract of *Leptadenia hastata* obtained on day 10 was 218.07 mg/kg. This inhibitory action of the different doses of the extract on *Trypanosoma congolense* parasites could be explained by the presence of active substances such as alkaloids, which have antiparasitic activity [21]. This activity could also be explained by the trypanocidal activity of alkaloids and flavonoids. Mamoudou [7] have shown that the accumulation of diminazene rapidly and specifically in the kinetoplast, a parasite organelle containing DNA,
suggests selective inhibition of parasite DNA synthesis.

The effect of the ethanolic extract on the parasite density of the *Leptadenia hastata* extract was relative to the days of treatment and the doses administered. The decrease was progressive over time. The 1000 mg/kg dose induced a significant decrease compared to the doses (250 and 500 mg/kg). This effect is comparable to that of the 1000 mg/kg dose revealed by isometamidium chloride. Indeed, whatever the concentration used the parasite densities decrease when the time increases. This decrease would be due to the concentration of the active principle [22]. We can say that this decrease is caused by the dilution, which decreases the effectiveness of the extract. The less active effects of the 250 and 500 mg/kg doses are comparable to those obtained by Vitouley [20] on the study of the trypanocidal potential of aqueous plant extracts for the treatment of trypanosomiasis. He showed that some plants used at a low dose, have a weak action.

Variations in the mortality rate of parasites by the effect of the ethanolic extract on the tenth day (D_{10}) of treatment induced a mortality rate higher than 50 %. The activity of this extract could be explained by its composition: alkaloids, tannins, terpenes and flavonoids that have antiparasitic activity [23]. Parasite mortality on the tenth day of treatment was increasing with increasing doses. Ethanolic extract of *Leptadenia hastata* induced a mortality of 59.18% at a dose of 1000 mg/kg. These results are statistically comparable to the 85% mortality rate induced by the isometamidium chloride positive control. The lethal dose 50 of ethanolic extract of *Leptadenia hastata* was obtained at 229.07 mg/kg on day 10 (D_{10}), which could be explained by the presence of active substances such as alkaloids with anti-parasitic activity.

CONCLUSION

At the end of this study, it was found that extracts of *Leptadenia hastata* showed trypanocidal activity, which confirms the concern of farmers about the decline in animal fertility after consumption of this plant. Thus, we noted mortality rates of 48%, 68.75% and 59.18% respectively for doses of 250; 500 and 1000 mg/kg of the ethanolic extract of *Leptadenia hastata* while the aqueous extract of *Leptadenia hastata* induced mortality rates of 35%, 60% and 41% respectively for the doses 250; 500 and 1000 mg/kg. The lethal doses 50 of ethanolic and aqueous extracts of *Leptadenia hastata* noted were 229.07 and 271.37 mg/kg on *Trypanosoma congolense* respectively.

Ethical Approval:

As per international standard or university standard ethical approval has been collected and preserved by the authors.

Availability of data and material

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