STM JOURNALS

International Journal of
Biochemistry and Biomolecules Research

Volume 2, Issue 2, 2024
July—December
DOI (Journal): 10.37591/IJBBR

https://journals.stmjournals.com/ijbbr

Research IJBBR

In vitro and *In vivo* Trypanocidal Activity of Methanolic Extract of *Lantana camara* leaves on *Trypanosoma brucei* in Laboratory Mice

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Abstract

In this study, the methanol extract of Lantana camara *leaves were tested for their trypanocidal activity in* vitro in *a microtiter plate and* in vivo *using male laboratory mice. Five different concentrations 10mg/ml, 20 mg/ml, 30 mg/ml, 40mg/ml, and 50mg/ml of the extract were prepared and the time taken to immobilize the parasites were recorded. The* in vitro *results showed that the leave extract at concentrations of 50 mg/ml had the most promising trypanocidal activity against* Trypanosoma brucei. *The* LD_{50} *value calculated from the result of the acute toxicity test was found to be 952 mg/kg. Based on this result, three different concentrations of the leave extract were prepared namely 200 mg/kg, 400mg/kg, and 800mg/kg body weight and administered to male laboratory mice in the* in vivo *studies. The result of the* in vivo *trypanocidal activity revealed that the methanol extract had no prophylactic effect against* Trypanosoma brucei. *However, in the curative group there was consistent parasitaemic suppression which was accompanied by prolonged time of survivals especially with treatment doses of 400mg/kg and 800mg/kg body weight daily administered intraperitoneally, even though the number of parasites were not totally wiped out within the 7 days of treatment. In the management of trypanosomiasis, the findings in this study are indicative of the trypanocidal potential of* Lantana camara. *Hence, we recommended that the extract of the leaves as well as other parts of this plant should be further evaluated for trypanocidal activities.*

Keywords: *Lantana Camara*, *Trypanosoma Brucei*, methanol extract, trypanocidal activity, *In vitro* and *In vivo*

INTRODUCTION

Trypanosomes have been around for over three hundred million years as microscopic unicellular protozoa that are ubiquitous parasites of insects, plants, birds, bats, fish, amphibians, and mammals. Trypanosomiasis is one of the most important protozoan diseases on the African continent [1, 2] caused by several species of blood and tissue dwelling protozoan parasite called Trypanosomes whose complex lifecycle alternate between insect vector and the mammalian host [3].

Generally, trypanosomes are associated with diseases in Africa and South America. African trypanosomiasis is commonly known as sleeping sickness in humans and Nagana (meaning “loss of spirit” in Zulu language) in cattle. The vectors responsible for this disease are tsetse flies and blood sucking reduvid bugs which transmit the trypanosomes either mechanically or biologically to humans, domestic and wild animals [4].

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Received Date: November 06, 2024
Accepted Date: November 21, 2024
Published Date: December 24, 2024

Citation: Ajaegbu Ernest Chinedu, Ezekiel Kogi. *In vitro* and *In vivo* Trypanocidal Activity of Methanolic Extract of *Lantana camara* leaves on *Trypanosoma brucei* in Laboratory Mice. International Journal of Biochemistry and Biomolecules Research. 2024; 2(2): 32–41p.

In west and central Africa, the causative agent of trypanosomiasis which affects humans is *Trypanosoma brucei* gambiense while *Trypanosoma brucei* rhodesiense is common in East Africa. *Trypanosoma brucei brucei* on the other hand is known to affect animals while *Trypanosoma cruzi* causes chagas disease which is a chronic human infection prevalent throughout Latin America, extending to the southern borders of U.S.A. [5].

The trypanosome species which are known to affect livestock in Africa are *T. brucei* brucei in cattle, *T. vivax* in sheep, *T. congolense* in goats, *T. simae* in pigs, and *T. evansi* in camels [6].

In Nigeria, the disease is prevalent especially in the northern part of the country and animals that are infected become weak, emaciate and abortion or infertility may occur in reproductively breeding animals leading to reduction in cattle yield production [7, 8]**.** This in turn may affect a part of the country's economy. The disease is known to constrain agricultural development in more than one third of the African continent.

Approaches in control of trypanosomiasis are chemotherapy, chemoprophylaxis and tsetse fly control [9, 10]**.** Over the years, some of the drugs which have been used primarily for the treatment of trypanosomiasis include Suramin, Tryparsamide, and Pentamidine among others [11–13]. Also, in the northeast part of Tanzania, prophylactic drugs have been used to control animal trypanosomiasis for over 30 years after which they developed resistance to the drugs [14, 15].

During pathogenesis, the host develops antibodies against the surface coat glycoprotein of the trypanosomes resulting in immune complexes [16, 17]. Interestingly, because of the possession of variant specific glycogotine genes (VSGs) coding for different surface coat proteins, the parasites subsequently evade these antibodies there by making the disease difficult to control medicinally [18].

Paul Ehrlich and Kiyoshi Shiga developed the first effective treatment which was Atoxyl, an arsenic based drug in 1910 but blindness was a serious side effect [19]. Numerous drugs have been designed to treat the disease which have been introduced since then and many researches have been carried out on the trypanocidal effect of plant extracts in the treatment of the infection which include the use of *Azadirachta indica* (Neem tree) [20], use of *Coptus afer* [21], use of Trypanocidal withanolides and withanolide glycosides [22] and the use of some Nigerian Savanna plants [23].

Justification

There are no vaccines and fully non-toxic modern drugs to treat diseases caused by trypanosomes. The few drugs available have their origin in the first half of the 20th century with issues of toxicity, complexity in administration of the drugs, high cost of purchase, and drug resistance development by the parasite. Hence the need for continuous investment and research in drug discovery with the aim of developing safer and more effective drugs [24].

Objectives

This research was carried out with the following objectives:

1. To determine the trypanocidal effects of methanolic leaf extract of *Lantana camara* on *Trypanosoma brucei in vitro*.
2. To determine the prophylactic effect of the extract at different concentrations *in vivo* using male laboratory mice.
3. To determine the curative effect of the extract at different concentrations *in vivo* using male laboratory mice.

MATERIALS AND METHOD

Plant Material

The leaves of *Lantana camara* which were the plant materials needed were collected from the Botanical Garden of the Department of Biological Sciences ABU Zaria. The identity of the plant was confirmed and a specimen stored in the herbarium section of the same department.

Plant Preparation

Three hundred and twenty grams of freshly collected leaves of *Lantana camara* were dried at ambient temperature to discourage enzymatic chemical reactions and growth of molds and bacteria in the leaves which could bring about undesirable changes in their constituents. After drying thoroughly, the leaves were garbled to remove extraneous matters and pounded using a mortar and pestle which were later sieved and stored in an enclosed container at room temperature [25].

Extraction of Plant Materials

The soxhlet extraction process was used to separate the chemical constituents of the plant using methanol solvent. Fifty (50) grams of air dried and pulverized plant was defatted using petroleum spirit at (60–80°C), using the soxhlet extractor. The defatted pulverized plant materials were extracted with absolute methanol at room temperature for 8 hours. To obtain the concentrated extract of the leaves, the methanol extract was filtered and the filtrate evaporated to dryness in a rotary evaporator. The final product was a solvent free extract which was poured in an enclosed vessel and stored in the refrigerator at a temperature of +4°C [26].

Experimental Animals

Fifty (50) laboratory mice which were all males of 4–5 weeks old were bought in cages from the animal house in Faculty of Pharmaceutical science Ahmadu Bello University Zaria and kept in a well-ventilated animal house where they were cared for and well fed with rat pellets and water regularly. The animals were acclimatized for 2 weeks before being used for the experiment which was conducted in accordance with the National Research Council guideline for care and use of laboratory animal [27].

***In Vitro* Trypanocidal Activity of Plant Extract**

The methanol leave extract was tested for trypanocidal activity *in vitro* which was carried out in a microtiter plate containing 96 conical bottom wells. Each well had a capacity of 0.25 milliliters equivalent to 250 microliters and a total of 18wells were used for the test [28]. As described by Nok et al. (1993) [20], five different concentrations of the extract were prepared which were 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, and 50 mg/ml.

Blood was collected from the infected mice using a syringe and needle into a sample bottle containing Ethylene Diamine Tetra acetic Acid (EDTA). Using a micropipette and separate tips, 20 μL of each concentration of the extract were dispensed into each well and 60 μL of heparinized infected blood was added to each well containing extract. Blood and extract were stirred immediately and a drop of the mixture was placed on a clean slide and covered with a cover slip. This was immediately placed under a compound microscope and observed under X40 objectives lens. Also, the time taken for the extract to completely stop the movement of the parasite was carefully monitored, recorded, and the same process repeated in triplicate for each concentration of the extract. Control wells without the extract contained a mixture of 20 μL of phosphate buffer saline and 60 μL of infected blood were also examined at the same time intervals with the experimental groups [29–37].

Acute Toxicity Test

A total of 13 mice were used for the toxicity test. As described by Dietrich Lorke [38], the acute toxicity of the plant extract to be investigated was tested in two stages. In the first stage, the range of doses producing the toxic effect was established and based on the results obtained, further specific doses were administered to obtain the LD_{50}.

In the first stage, a total of 9 mice were divided into three groups namely A, B, & C with 3 members in each group. These were given differing doses of 1000 mg/kg, 100 mg/kg, and 10 mg/kg body weight respectively by the intraperitoneal route using a 1ml insulin syringe and needle. The animals were then monitored for few days for signs of discomfort and final death.

The second stage was based on the result of the first stage in which further doses of 1600 mg/kg, 800 mg/kg, 400 mg/kg, and 200 mg/kg body weight were administered to 4 mice (A_2, B_2, C_2 and D_2) which were also monitored for few days for signs of discomfort and death. The result obtained were used in computing the LD_{50}.

Pack Cell Volume (PCV)

The tail of each mouse was cut using scissors and blood was taken to three-quarter the level of a capillary tube and one end of the tube sealed using a Bunsen burner. The tubes were placed in a hematocrit centrifuge and allowed to spin for 3mins after which each capillary tube was read and recorded. This was done for each mouse before inoculation of parasite, after inoculation at massive parasitemia as well as during and after treatment.

Parasite Inoculation into Animals

About 0.4 ml of blood was taken from the tail of a donor mouse with massive parasitemia and diluted with 0.6 ml of normal saline. A total of 20 mice were divided into 5 groups namely A, B, C, D, & E. Members of group A, B, C & D were inoculated with 0.1ml of diluted blood intraperitoneally using a 1ml syringe and needle. Group D served as the positive control and group E with no infection served as the negative control. The levels of parasitemia in infected mice were then monitored daily until massive parasitemia was attained in each mouse.

Trypanocidal Activities of Plant Extract *In Vivo*

Three different concentrations of the extract; 800 mg/kg, 400 mg/kg, and 200 mg/kg body weight were prepared based on the results obtained from the acute toxicity test and where each tested for their trypanocidal effects. The various concentrations were administered by the intraperitoneal route to groups A, B, & C respectively using a 1ml syringe and needle. The treatment of infected mice for a period of 7days was initiated 3 days post infection after experimental groups had attained a high level of parasitemia. Group D were infected mice that were not treated thus serving as the positive control. Daily parasite count was done until the positive control mice group died.

Another set of 20 mices were again divided into 5 groups A, B, C, D, & E to test for the prophylactic effect of the extract *in vivo* and were also administered 800 mg/kg, 400 mg/kg, and 200 mg/kg body weight of the extract with group D & E serving as positive and negative controls respectively.

In these groups, treatment was administered intraperitoneally first for two days before the parasites were inoculated into the mice and daily parasite count was done until the positive control mice group died.

Daily Parasite Count

As described by Herbert and Lumsden (1976) [29], blood was collected from the tail of an infected mouse of which a drop was placed on a clean slide and covered with a cover slip. The slide was placed under a binocular microscope and observed at X400 objective lens. The microscopic appearance of the wet film of infected blood was matched with one of a series of eight pictures of microscopic fields. Parasite counts were matched with the computation chart to calculate the logarithm number of parasites and subsequently the number of parasites in 1ml of infected blood. This was carried out for each mouse daily.

RESULTS

The *in vitro* test of varying concentrations of the methanol extract of *L. camara* on *T. brucei* motility showed that the time taken for the elimination of motility was inversely proportional to the concentration of the extract. The lowest concentration of 10 mg/ml had the longest motility with elimination time of 56 minutes while the highest concentration of 50 mg/ml had the lowest time of

22mins. However, in the untreated wells (control), *T. brucei* remained motile even after 90 mins of trypanocidal studies (Table 1).

Table 1. The effect of methanol leaves extract of *L. camara* on *T. brucei in vitro.*

Experimental Groups	Concentration (mg/ml)	T_1 (mins)	T_2 (mins)	T_3 (mins)	Average Time (mins)
A	10	50	55	64	56
B	20	40	46	56	47
C	30	32	33	34	33
D	40	30	32	33	31
E	50	20	23	25	22
Control	0	90	90	90	90

Statistical analysis using ANOVA showed significant differences in the different times taken to immobilize *Trypanosoma brucei* with the different concentrations of the extract. The different means of the time taken to immobilize Trypanosomes for the three different timings and concentrations of the extract were further separated using Duncan's multiple range test. But the extract at concentration of 30mg/ml and 40 mg/ml was not significantly different. From the acute toxicity test carried out based on the Dietrich Lorke method an LD_{50} value of 952 mg/kg was calculated (Table 2).

Table 2. Acute toxicity test of methanol extract (first stage of the test).

Experimental Group (1)	Weight (g)	Dose (mg/kg)	Volume (ml)	Time of Death (days)	No. of Death / No. Used
A1	18.5	1000	0.19	1	
A2	19.4	1000	0.20		
A3	19.0	1000	0.19	2	2/3
B1	18.9	100	0.19		
B2	20.6	100	0.21		
B3	19.3	100	0.19		0/3
C1	20.8	10	0.21		
C2	25.0	10	0.25		
C3	19.2	10	0.19		0/3

Table 2. (Second Stage of the test).

Experimental Group (2)	Weight (g)	Dose (mg/kg)	Volume (ml)	Time of Death (days)	No. of Death / No. Used
A_2	18.3	1600	0.18	1	1/1
B_2	19.8	800	0.20		0/1
C_2	17.6	400	0.18		0/1
D_2	16.0	200	0.16		0/1

Lethal Dose (LD)

LD_{50} = 952 mg/kg

In the prophylactic group in which members were treated first before being infected with *T. brucei*, the methanol extract at doses of 800 mg/kg, 400 mg/kg, and 200 mg/kg revealed no preventive effect since the examination of blood of infected mice by rapid wet mount method showed high level of parasitaemia 3days post infection.

The *in vivo* trypanocidal studies within the 7 days of treatment with methanol leave extracted at doses of 800 mg/kg and 400 mg/kg body weight revealed a drastic reduction in the level of parasitemia with prolonged time of survival of infected mice. On the contrary, the extract at a dose of 200 mg/kg had no effect on the parasites followed by increase in number of parasites in infected mice

which resulted in death 5 days post treatment. Members of the positive control group which were infected but untreated showed massive parasitemia with eventual death 6days post infection while the negative control group remained healthy (Figure 1).

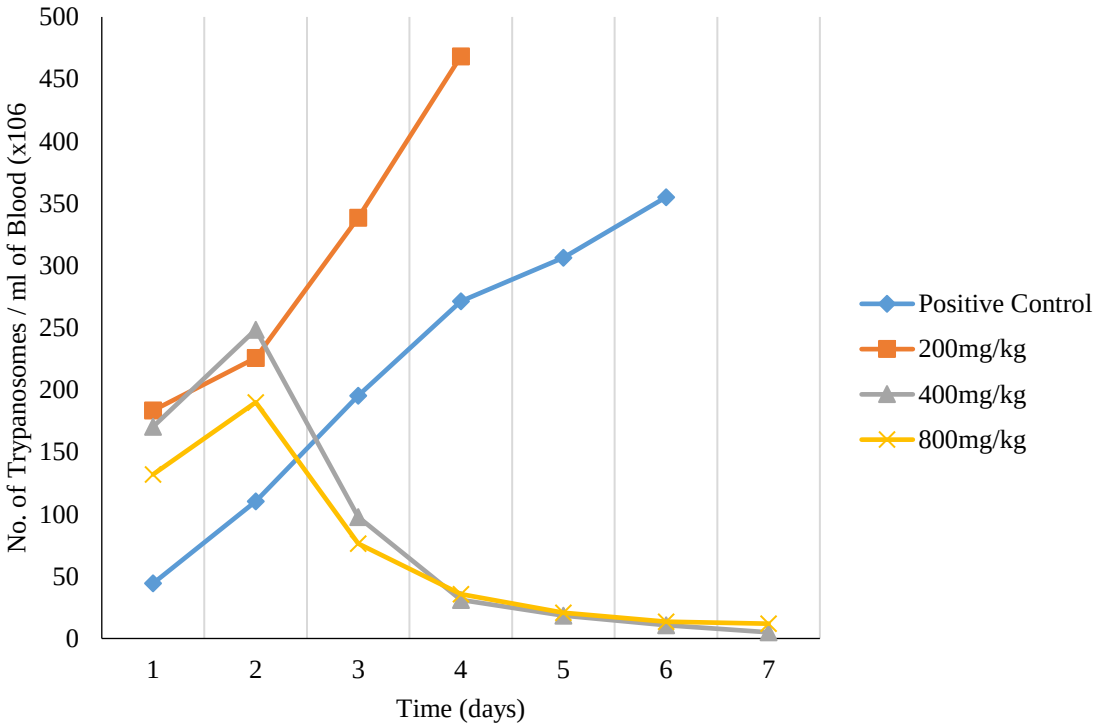

Figure 1. Daily parasites count during massive parasitaemia in *in vivo* trypanocidal studies of methanol extract of *L. camara* leaves.

Table 3. Number of Trypanosomes per ml of blood $X10^6$ during massive parasitemia.

Days	**Positive Control**	**200 mg/kg**	**400 mg/kg**	**800 mg/kg**
1	44.3	183.4	170.3	132
2	110.3	225.8	248.5	190
3	195.3	338.5	97.8	76.35
4	271.3	468.3	30.9	35.75
5	306.3	Dead	18.5	20.7
6	355	–	10.6	13.4
7	Dead	–	4.98	11.9

The trypanocidal activities of the extract at doses of 400 mg/kg and 800 mg/kg were obvious compared to the positive control group (Table 3). The results which were subjected to statistical analysis of ANOVA showed significant differences at different levels of parasitemia with the varied doses of methanol extract during massive parasitemia levels. The different means of parasitemia at the different days and doses of the extract separated using Duncan's Multiple Range test revealed that Group C and D given 200 mg/kg and 0 mg/kg dose of the extract respectively were not significantly different, and Group A and B given 800 mg/kg and 400 mg/kg dose, respectively were not significantly different as well (Table 4).

PCV readings analyzed statistically using ANOVA showed no significant differences with the different doses of the extract. However, there was noticeable improvement in the PCV levels after treatment for groups A and B given doses of 800 mg/kg and 400mg/kg respectively (Table 5).

Table 4. Duncan's multiple range test for *In vivo*.

Duncan Group	Mean	Number of Observations	Day
A	201.54	16	4
A	193.82	16	2
A	176.97	16	3
B	132.44	16	1
B	115.16	12	5
C	77.40	15	6
D	5.18	13	7

Table 4. (In Groups.).

Duncan Group	Mean	Number of Observations	Groups
A	181.57	28	C
A	171.08	28	D
B	82.10	32	B
B	67.24	32	A

Note: Means with the same letters are not significantly different.

Table 5. Mean pack cell volume readings for curative group.

Experimental Groups	Weight (g)	PCV Before Inoculation	PCV at Massive Parasitaemia	PCV During Treatment	PCV After Treatment
A	23.5	45	31	30	37
B	26.0	46	33	35	40
C	23.0	44	36	31	Dead
D	24.0	44	33	28	Dead
E	23.0	45	45	45	45

DISCUSSION

The results obtained showed that the extract of the leaves of *Lantana camara* possessed trypanocidal activities against *T. brucei*. Although this study did not involve structure elucidation, but reports reveal that extracts showing potent trypanocidal activity contain alkaloids, flavonoids, terpenes, or arthraquinones [30–32].

The difference observed in the trypanocidal activity of the different concentration of the extract may have resulted from the difference in the concentrations of the active component of the extract. Comparing the *in vitro* with the *in vivo* experiment, trypanocidal activity was high in contrast to the results obtained from the *in vivo* studies. This is in line with similar experiments [33, 34] which explained that the difference may likely be since in a multicellular organism metabolic transformation of active molecules to inactive ones, or vice versa, may occur and might thus lead to change in activity under *in vivo* conditions.

The intraperitoneal route of administration of extract was preferred to the oral route in that it is known to be faster in absorption and effect [35]. From the results obtained from the Duncan's multiple range test for *in vivo* studies which indicated that the extract at dose 800 mg/kg and 400 mg/kg were not significantly different in their effects on *Trypanosoma brucei*, it meant that the effective dose of the extract was 400mg/kg and increasing the dose would not increase the effect produced instead the toxicity of the extract is been increased since the LD_{50} is 952 mg/kg. Hence, any dose close to this value or greater could be detrimental to the animal. The extract at dose 200 mg/kg and the positive control group were not significantly different in that mice treated with 200 mg/kg dose still died almost the same time with the untreated mice but the treated had a higher tolerance

range and so were able to harbor greater number of parasites before their death than the untreated mice. Also, Duncan's multiple range test indicated that the extract at dose of 400 mg/kg and 800mg/kg were significantly different from the extract at dose of 200 mg/kg and the positive control group in their effects on *T. brucei*.

The consistent reduction in parasitaemic level combined with prolonged time of survival of mice given dose of 400 mg/kg and 800 mg/kg as shown in the *in vivo* results may be linked to the ability of the extract to inhibit glycolysis which is the major source of energy for blood stream *Trypanosoma brucei*.

While investigating the efficacy of the extract, there were variations in the administration of treatment of which treatment was administered first for 2 days before inoculation of the parasite (prophylactic group) and the other group which were inoculated with the parasite before treatment began after the parasites had been established in the host (curative group). However, in the prophylactic group, the extract could not prevent the establishment of the parasite in the host. This might have resulted from the inadequacy of the concentration of the active component of the extract in the host tissues, the variable surface glycoprotein of the trypanosomes or the distribution of the parasites in the mice.

Also in the curative group, the number of established parasites were drastically reduced within the days of treatment but not completely wiped out. This may be explained by the pathogenesis of Trypanosomes as they tend to migrate from the site of infection to the blood stream and the lymphatic system at the early phase of the disease where they proliferate [36]. At this stage, virulence can be decreased by administering specific drugs to modify the course of infection in the host. But at the late phase of the disease involving the nervous system with the parasites invading the brain and cerebrospinal fluid, most trypanocides available are seldom effective [37].

CONCLUSIONS

In conclusion, this research demonstrates that methanol leaf extract of *Lantana camara* exhibits trypanocidal activity *in vitro* against *T. brucei*. Also, at doses of 400 mg/kg and 800 mg/kg body weight, the extract holds some trypanocidal potentials in experimentally infected mice with no prophylactic effect *in vivo*. It is therefore recommended that the leaves of this plant be further evaluated including the use of a different solvent. In addition, other parts of the plant other than the leaves, such as the root, stem or bark should be assessed for trypanocidal activities.

Acknowledgment

The author wishes to express his gratitude to God Almighty for his faithfulness and the gift of life. He is also grateful to his Parents Mr. Sunday and Mrs Love Ajaegbu for their supports and encouragements.

Declaration of Interests

The author has nothing to declare.

Funding Sources

This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

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