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In Vitro And *In Vivo* Assessment of Theantitrypanosomal Activity of Methanol Leaf Extract of *Annona Muricata*(Meam) on the Blood Parameters Of *Trypanasoma Brucei Brucei* Infected Albino Rats.

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***ABSTRACT*-This study investigated the anti trypanosomal activity of the methanol leaf extract of *Annona muricata* [MEAM*]* in albino rats experimentally infected with *Trypanosoma brucei brucei.* A total of 32 adult male albino rats weighing between 78g and 100g were used for the study. They were randomly assigned into 4 groups of three rats each for the acute toxicity study and 4 groups [I-IV] of 5 rats each for the *In vivo* study. Groups I and II were infected intraperitoneally with 1.0 $\times 10^6$ trypanosomes suspended in 0.2ml Phosphate Buffered Saline[PBS]on day 8 of experiment. Group I was treated with 200 mg/kg of MEAM orally every day for 3 days following detectable parasitaemia [4-5days of post infection]. Group II was treated with 7.0 mg/kg of diminazene aceturate intramuscularly on day 14 of experiment [6 days post infection]. Group III served as the uninfected untreated control while Group IV was uninfected and treated orally with 200mg/kg MEAM. Parameters such as Parasitaemia, Packed cell volume [PCV], Hemoglobinconcentration, Total leucocytes count, Differential leucocytes count. The acute toxicity test and the *in vitro* anti trypanosomal activity of the extract were studied using standard methods prior to commencement of the study. The results of the acute toxicity test showed the LD_{50} of the MEAM to be 447 mg/kg whereas the LC_{50} of the extract was recorded at 0.01mg/ml*in vitro*. The pre patent period of infection was 3days. Treatment with diminazene aceturate cleared the Parasitaemia in group II. An overall significant decrease[$P< 0.05$] in PCV and Hemoglobin was observed in the infected groups. Similarly, a significant decrease [$P< 0.05$] in total leucocytes count was also seen in the infected groups. Treatment with MEAM showed increased Parasitaemia, indicating that MEAM showed an *In vitro* anti trypanosomal activity and no anti trypanosomal activity *In vivo*. It is recommended that parental routes of administration of MEAM should be employed for further studies of anti trypanosomal activity of MEAM.**

***Keywords*- *Trypanosomal brucei brucei*, methanol leaf extract, *Annona muricata*, blood parameters, albino rats.**

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I. INTRODUCTION

Animal trypanosomosis caused by trypanosomes has been one major cause of morbidity and mortality of livestock in sub-saharan Africa, therefore causing serious losses in cattle, pigs, camel, goats and sheep [16].The disease which is transmitted by tsetse flies is caused by *Trypanosoma congolense, Trypanosome vivax* and *Trypanosoma brucei brucei*. Human African trypanosomosis is caused by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense.* The other form of Trypanosomosis called Chagas disease causes an estimated 21,000 deaths per year and occurs mainly in Latin America [17].

Currently, approximately 60million people and approximately 48million cattle are at risk of this disease in an area of 10million square kilometers. It is also responsible for 5000 humans and 3million livestock deaths annually [28], [13], [10], [1], [18]. The diseases transmitted by tsetse fly mainly of the *GenusGlossina* represent the most debilitating on human development, agricultural production, food security and sustainable human livelihood on the continent with an estimated loss in livestock production and mixed agriculture in Africa valued at 5billion US dollars yearly[29], [2], [24].

It is nearly 100 years of tsetse and trypanosomosis control efforts, but today the problem is still far from being solved. In most African countries, tsetse distribution has remained the same and indeed in some areas, the fly has spread to new areas. The incidence of both animal and human trypanosomosis remained high with occasional endemic outbreak [21]. There has been extensive international collaboration and extensive expenditure on integrated mechanisms to control the disease and its vector. In 1974, the Food and Agricultural Organization (FAO) was given the United Nations (UN) mandate to co-ordinate the eradication of tsetse (*Glossina spp*) and trypanosomosis from Africa, in ten years. The disease which is commonly known as Nagana embraces a variety of clinical manifestations, but usually involves fevers, headaches, anemia, anorexia and wasting.

Trypanosomosis is suspected when an animal in an endemic area is anemic and in poor condition [20]. Trypanosomosis is recognized as one of the major diseases of economic importance to humans and domestic animals in Africa. Chemotherapy is one of the means of controlling the disease. Currently, four drugs (pentamidine,suramin, melarsoprol and eflornithine) are available for treatment of human African trypanosomosis while five drugs (diminazene,isometamidium, homidium bromide and chloride and quina pyramine compounds) are used for the treatment of animal Africantrypanosomosis. These drugs used for the treatment of trypanosomosisboth in animals and man are beset with challenges of severe toxicity and increasing incidences of trypanosome resistance and relapse [22]. This situation suggests the need to develop newer, easily available and safer trypanocides [7], by exploring the potentials of efficacious chemotherapeutic agents from locally available medicinal plants.

The methanol leaf extract of *Annona muricata* [MEAM] has shown activity against malaria parasites [3]. Phytochemical screening revealed

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that *Annona muricata* stem bark extract is rich in flavonoids and tannins such as annonaceous acetogenins, annocatalin, annohexocin, annomonicin, muricapentocin, xylomaticin etc. with the above-named activities of this plant and the potential for more, it is therefore thought that the antitrypanosomal potential of this plant be investigated with a view to finding alternative chemotherapeutic solutions to trypanosomosis, hence the needfor this study. The objective of this study is to investigate the antitrypanosomal potential of the methanol leaf extract of *Annona muricata* (MEAM) on the blood parameters of *T. brucei brucei* infected albino rats.

II. MATERIALS AND METHODS

Thirty-two(32) adult male albino rats weighing between 78g to 100g were used for the study. The rats were purchased from the Faculty of Veterinary Medicine, University of Nigeria Nsukka. They were randomly assigned into four (4) groups of 3 rats each for the acute toxicity study and another 4 groups of five (5) rats each for the *In vivo* study. They were identified with picric acid body markings and kept in clean metal cages in the laboratory animal house of the Department of Veterinary Parasitology and Entomology. The rats were acclimatized for three weeks before the commencement of the experiment. They were dewormed with albendazole (Zolat^{R}) for gastro intestinal parasites and screened for presence of blood parasites. They were provided clean water and fed standard rat feed *ad libitium*.

Trypanosomes used in the study were originally isolated from a pig presented with clinical trypanosomosis at the University of Nigeria Veterinary Teaching Hospital. It was identified morphologically and using laboratory animal inoculation to be *Trypanosoma brucei brucei*. They were maintained in mice which served as the donors for inoculation of the recipient experimental rats.

The plants *Annona muricata* was obtained from the Department of Crop Science, Faculty of Agriculture, University of Nigeria Nsukka. It was extracted in the Department of Veterinary Physiology and Pharmacology, Faculty of Veterinary Medicine, University of Nigeria Nsukka using the cold maceration method [15] with 80% methanol. After extraction, the percentage yield was calculated using the following formula.

$$\% \text{ yield} = \frac{\text{weight of extract}}{\text{Original weight of plant}} \times \frac{100}{1}$$

Where weight of extract = 8.7g

Original weight of plant = 300g

Percentage yield = 2.9%

The twenty [20] rats were divided into 4 groups as follows;

Group 1: Infected and treated with *Annona muricata* extract at 200mg/kg orally

Group 2: Infected and treated with diminazene aceturate at 7.0mg/kg I/m

Group 3: Uninfected and untreated (negative control)

Group 4: Uninfected and treated with *Annona muricata* at 200mg/kg orally.

Each rat in groups 1 and 2 were inoculated intraperitoneally with 1.0×10^{6} trypanosomes suspended in 0.2 ml of phosphate buffered saline (PBS)The rats were screened from the 3rd day post infection using wet mount method to establish the onset and level of parasitaemia. The following

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parameters were investigated to assess the efficacy of the extract; parasitaemia, packed cell volume, haemoglobinconcentration, total leucocytes count and differential leucocytes count.
For the acute toxicity study; Different doses of the *A. muricata* extract were used for the different groups. The rats in each group were given the respective doses of the extract according to the body weight and experimental design. The rats were observed for a period of twenty-four(24) hours, after which a graph of percentage (%) mortality of the rats against Log dose of the extract was plotted, and the slope determined. The absolute value of the slope is the LD_{50}.

The *In vitro* assay of the extract involved 98 wells in the micro titre plate with different concentrations of the extract of *A. muricata* in double serial dilutions. Micropipette was used to collect a constant volume of *T. brucei brucei* infected blood and then introduced into the different wells. The efficacy of the extract against the *T. brucei brucei* was checked every five (5) minutes. In a situation where there is no activity of the extract on the *T. brucei brucei,* a further five (5) minutes is given at that well, this is done till either activity of the extract on *T. brucei brucei* is observed or after one (1) hour and there is no activity then the concentration of the preceding well is the least concentration (LC_{50}) where the extract is active against *T. brucei brucei*

Blood was collected from the tail vein by nipping the tip of the tail with a pair of scissors. A wet mount was then made by milking the tail, placing a drop of blood on clean grease free microscope slide and placing a clean cover slip over the drop of blood so that the blood spreads evenly. This was then examined carefully under the microscope for trypanosomes using ×40 objective. The level of parasitaemia was then estimated using the Rapid matching method by Herbert and Lumsden (1976) This involves matching the microscope field in focus with one of a series of eight pictures of microscopic fields. The best match was then selected and the number of trypanosomes recorded as logarithm of number in base 10. This was done from the 3rd day post–infection until the end of experiment.

Estimation of PCV was done using the microhematocrit method (Coles, 1986)Blood was collected from the retro-orbital plexus of the median cantus of the eye into heparinized sample bottles. Heparinized capillary tubes were used to collect blood from the sample bottles up to three quarter full. One end was sealed with plastacine and centrifuged with a microhaematocrit centrifuge (Hawksley, England) for 5 minutes at 10,000 revolutions per minute (rpm)The PCV was then read as a percentage using a microhaematocrit reader (Hawksley, England)

The Heamoglobin concentration was determined using the cyanohaemoglobin method (Schalm, *et al*1975),5mls of Drabkins reagent was put in a clean test tube twenty (20ul) microliter of blood was added to the reagent and mixed properly. The mixture was then allowed to react for 20 minutes and the absorbance was read at 540nm wavelength against a reagent blank on a colorimeter (Lab-tech, India)

The white blood cells were counted using the improved Neubauer chamber (Coles 1986)Three

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hundred and eighty (380ul)micro liter of WBC diluting fluid was added to the test tubes in which 20ul of blood was equally added and mixed properly. The counting chamber was charged with the diluted cells, mounted on a microscope and allowed to settle. The cells were then viewed using x10 objective lens and counted in the 4 corner squares and values.

III. RESULTS

The results of the acute toxicity tests are shown below;

GROUPS	NO OF AN	NO DEAD	LOG DOSE	% MORTALITY		
100mg/kg	3	0	2	0		
200mg/kg	3	0	2.3	0		
400mg/kg	3	1	2.6	33.33		
800mg/kg	3	3	2.9	100		

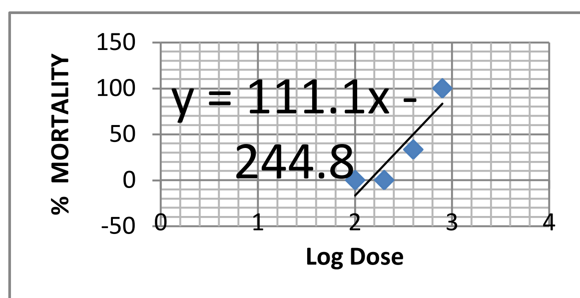

Fig 1: A graph of % mortality against Log Dose, where the value of X, after substituting 50 for Y signifies LD_{50} of MEAM

Substitute 50 for Y in the equation y = 111.1 x — 244.8 and solve for X, the antilog of the value of X is the LD_{50}

$$50 = 111.1x - 244.8$$

$$50 + 244.8 = 111.1x$$

$$294.8 = 111.1xe$$

$$X = 294.8/111.1$$

$$X = 2.65$$

Antilog of 2.65 = 447mg/kg

The last concentration of Methanol leaf extract of *Annona muricata* which showed activity against trypanosomes is the LC_{50}. From Table I 0.01mg/ml is the LC_{50} of Methanol leaf extract of *Annona muricata*

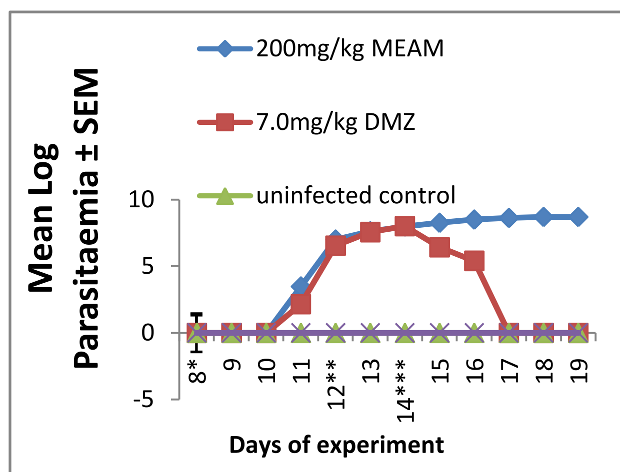

Fig 2: Mean Log Parasitaemia of rats infected with *T. brucei brucei* and treated with MEAM and diminazene aceturate.

8*- day of infection of Group 1 and Group 2

12**- day of treatment of Group 1 with 200mg of MEAM

14*- Day of treatment of Group 2 with Diminazene aceturate

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Fig 2 showed mean log parasitaemia. The infected groups (group 1 and group 2), showed detectable parasitaemia on day 3 post infection (corresponding to day 11 of experiment) The parasitaemia continued to increase in both groups until day 7 post infection (day 15 of experiment), when there was a drop in parasitaemia in group 2 following treatment with 7.0mg/kg of Diminazene aceturate. This decrease in parasitaemia continued in group 2 until day 9 post infection (day 17 of experiment) when the parasitaemia cleared. However, the level of parasitaemia continued to increase in group 1 regardless of the treatment with 200mg/kg of MEAM.

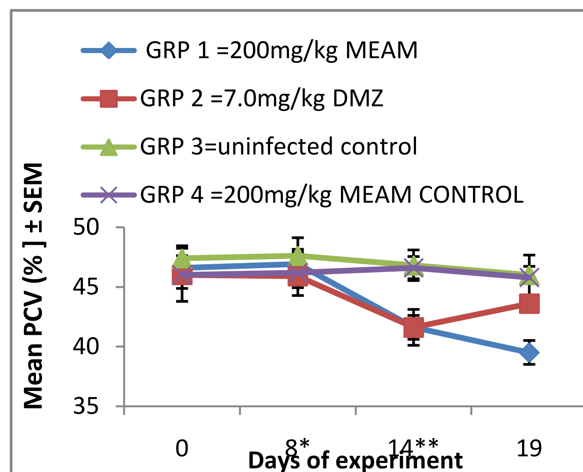

Fig 3: Mean PCV of rats infected with *T. brucei brucei* and treated with MEAM and diminazene aceturate.

8*- day of infection of Group 1 and Group 2

14**- treatment of Group 2 with 7.0mg/kg Diminazene aceturate/ treatment with 200mg/kg MEAM end.

Fig 3 shows the mean PCV. Following the infection of rats in groups 1 and 2 with *Trypanosoma brucei brucei* on day 8 of the experiment, themean PCV of rats in these groups was significantly lower ($P < 0.05$) than those of rats in groups 3 and 4 which were the uninfected/untreated control and the 200mg/kg MEAM/uninfected groups respectively. There was a significantly higher ($P <0.05$) mean PCV of rats in group 2 (following treatment with 7.0mg/kg diminazene aceturate on day 14 of the experiment) than in group 1 (regardless of treatment with 200mg/kg of MEAM from days 12 – 14) There was no significant change ($P >0.05$)in the mean PCV of rats in group 3 and group 4 throughout the experiment.

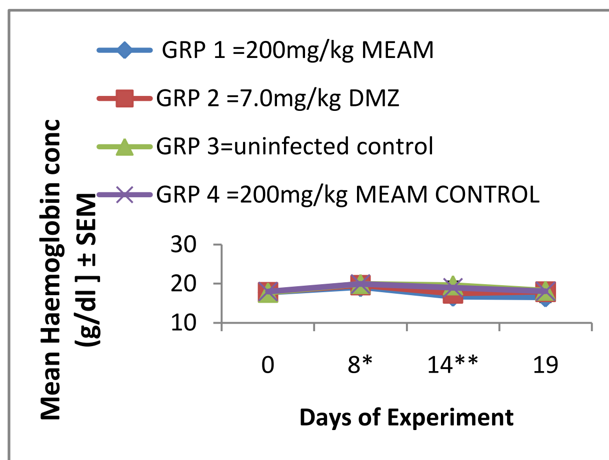

Fig 4: Mean Haemoglobin concentration of rats infected with *T. brucei brucei* and treated with MEAM and diminazene aceturate.

8*- day of infection of Group 1 and Group 2

14**- treatment of Group 2 with Diminazene aceturate/ treatment with 200mg/kg MEAM end

Fig 4 shows the mean haemoglobin concentration. Following infection of rats in groups 1 and 2 on day 8 of experiment, the mean haemoglobin concentration was significantly lower ($P < 0.05$)than the mean haemoglobin concentration of

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rats in groups 3 and 4. The mean haemoglobin concentration of rats in group 2 (following treatment with 7.0mg/kg of diminazene aceturate on day 14 of the experiment) was significantly higher (P<0.05) than the mean haemoglobin concentration of rats in group 1 [despite treatment with 200mg/kg of MEAM. There was no significant difference (P < 0.05) in the mean haemoglobin concentration of rats in groups 3 and 4 throughout the experiment.

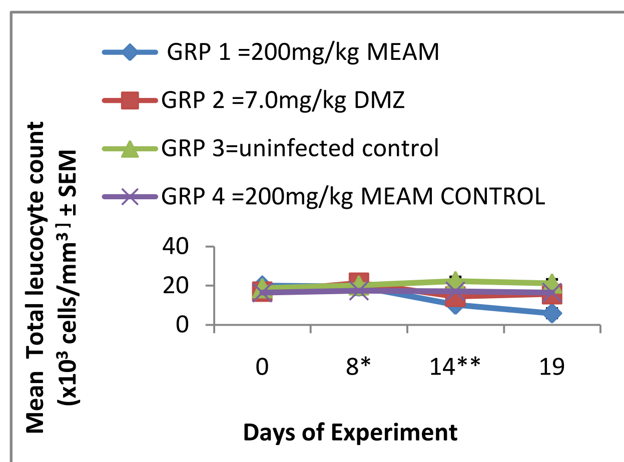

Fig 5: Mean Total Leucocyte count of rats infected with *T. brucei brucei* and treated with MEAM and diminazene aceturate.

8*- day of infection of Group 1 and Group 2

14**- treatment of Group 2 with Diminazene aceturate/ treatment with 200mg/kg MEAM end

Fig 5 shows the mean Total leucocyte count. Following the infection of rats in groups 1 and 2 (on day 8) the mean total leucocyte count was significantly lower (P <0.05) than the mean total leucocyte count of rats in groups 3 and 4. The mean total leucocyte count of rats in group 2 was significantly higher (P < 0.05) than those of group 1 on day 19 [12 days post infection] following treatment of rats in group 2 with 7.0mg/kg of diminazene aceturate. There was no significant difference (P <0.05) in the mean total leucocyte count of rats in groups 3 and 4 throughout the experiment.

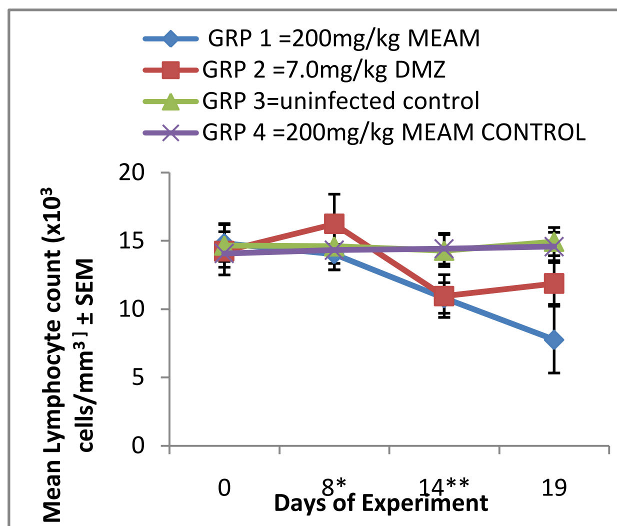

Fig 6: Mean Lymphocyte count of rats infected with *T. brucei brucei* and treated with MEAM and diminazene aceturate.

8*- day of infection of Group 1 and Group 2

14**- treatment of Group 2 with Diminazene aceturate/ treatment with 200mg/kg MEAM end.

Fig 6 shows the mean absolute lymphocyte count. Following the infection of rats in groups 1 and 2, there was a significantly lower (P < 0.05) mean absolute lymphocyte count of rats in group 1 and group 2 than rats in groups 3 and 4. The mean absolute lymphocyte count of rats in group 2 (following treatment with 7.0mg/kg of diminazene aceturate) was significantly higher (P < 0.05) than those of rats in group 1 (despite treatment with 200mg/kg of MEAM) There was no significant

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difference (P < 0.05) in the mean absolute lymphocyte count of rats in group 3 and group 4 throughout the experiment.

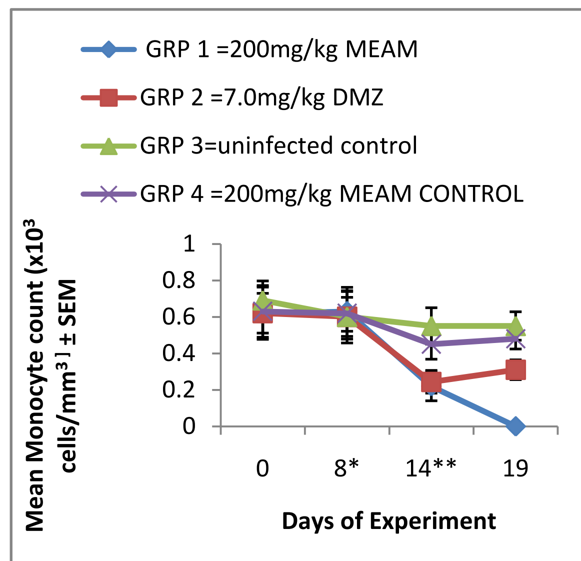

Fig 7: Mean Monocyte count of rats infected with *T. brucei brucei* and treated with MEAM and diminazene aceturate.

8*- day of infection of Group 1 and Group 2

14**- treatment of Group 2 with Diminazene aceturate/ treatment with 200mg/kg MEAM end.

Fig 7 shows the mean absolute monocyte count. Following infection on day 8, there was a significantly lower (P < 0.05) mean absolute monocyte count of rats in groups 1 and 2 than those of groups 3 and 4. There was a significantly higher (P < 0.05)mean absolute monocyte count of rats in group 2 following treatment with 7.0 mg/kg of diminazene aceturate) than those of group 1 (following treatment with 200mg/kg MEAM) There was no significant difference (P < 0.05) in the mean absolute monocyte count of the rats in groups 3 and 4 throughout the experiment.

MEAM concentrations (mg/ml)

TIME (MINS)	**CTRL**	**5.6**	**2.8**	**1.4**	**0.7**	**0.35**	**0.18**	**0.08**	**0.04**	**0.02**	**0.01**	**0.005**
0	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
5	++++	X	X	X	X	+++	+++	+++	+++	+++	+++	+++
10	++++	X	X	X	X	+++	+++	+++	+++	+++	+++	+++
15	++++	X	X	X	X	X	+++	+++	+++	+++	+++	+++
20	++++	X	X	X	X	X	X	+++	+++	+++	+++	+++
25	++++	X	X	X	X	X	X	X	++	++	++	+++

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30	++++	X	X	X	X	X	X	X	X	X	++	+++
35	++++	X	X	X	X	X	X	X	X	X	++	+++
40	++++	X	X	X	X	X	X	X	X	X	X	+++
45	++++	X	X	X	X	X	X	X	X	X	X	++
50	S	X	X	X	X	X	X	X	X	X	X	++
55	S	X	X	X	X	X	X	X	X	X	X	++
60	VS	X	X	X	X	X	X	X	X	X	X	++

Table I: Different concentrations of Methanol leaf extract of *Annona muricata* and the time taken for activity against trypanosomes *In vitro*

++++ = 4 parasites/field.

+++ = 3 parasites/ field.

++ = 2 parasites/field.

X = absence of parasite.

S = parasites seen but sluggish.

VS = parasites seen but very sluggish

Diminazene aceturate Concentrations (mg/ml)

TIME (MINS)	**CTRL**	**17.1**	**4.3**	**1.07**	**0.3**	**0.07**	**0.02**	**0.004**	**0.002**	**0.001**
0	++++	X	X	X	X	X	X	++++	++++	++++
5	++++	X	X	X	X	X	X	+++	+++	++++
10	++++	X	X	X	X	X	X	+++	+++	++++
15	++++	X	X	X	X	X	X	X	++	++++
20	++++	X	X	X	X	X	X	X	X	++++

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25	++++	X	X	X	X	X	X	X	X	++++
30	++++	X	X	X	X	X	X	X	X	++++
35	++++	X	X	X	X	X	X	X	X	++++
40	++++	X	X	X	X	X	X	X	X	++++
45	++++	X	X	X	X	X	X	X	X	++++
50	S	X	X	X	X	X	X	X	X	++++
55	S	X	X	X	X	X	X	X	X	++++
60	VS	X	X	X	X	X	X	X	X	++++

Table II: Different concentrations ofDiminazene aceturate and the time taken for activity against trypanosomes*In vitro*

++++ = 4 parasites/ field

+++ = 3 parasites/ field

++ = 2 parasites/field

X = absence of parasite

S = parasites seen but sluggish

VS = parasites seen but very sluggish

IV. DISCUSSION

The *In vitro* assay according to this study indicated that *Annona muricata,* has trypanocidal activity which have been reported to be due to the presence of bioactive compounds [22] The failure of the Methanol extract of *Annona muricata*(MEAM) to have trypanocidal activity *In vivo* may be due to its reduced bio-availability, absorption and metabolism. This could have accounted for the observed discrepancies between *In vitro* and *in vivo* tests [11] It is also probable that the routes of administration may also cause differences in results between *In vitro* and *In vivo* assays as has been observed with intra peritoneal and oral administration, this has been explained by the metabolic breakdown of compounds in the gastrointestinal tract or by lack of absorption into the blood stream [14]

Following the infection of the albino rats; an average pre-patent period of 4 days was recorded. Similar pre-patent period has been recorded in rats [9] The parasitaemia and anaemia recorded in the infected groups was typical of trypanosome infections in rodents [26] Parasitaemia was cleared only in group 2 following the administration of diminazene aceturate while that of the MEAM

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treated group continued to increase, this suggested a failure of the extract to inhibit the multiplication of the parasites in vivo.

The reductions in the PCV and haemoglobin among the infected groups were due to the anaemia caused by the trypanosome parasite and this is in line with several reports [8], [12], [27].These parameters returned to pre- infection values only in group 2; this may be due to the disappearance of the parasite from peripheral circulation leading to a reversal in the adverse effect of the parasite to the blood cells [23] However, the return of PCV and Haemoglobin concentration to pre- infection values was not observed in the MEAM treated group, which indicated progression of the disease despite treatment with MEAM.

Leucopoenia in the infected groups as shown in this study is because of immunosuppression, an important attribute of trypanosomosis and have been severally reported [4], [5] in infected animals. Leucopoeniacontinued in the MEAM treated group as the study progressed while the diminazene aceturate treated group showed a return of the leucocytes to the pre- infection values. This suggests that the extract could not stop the immunosuppression caused by the multiplication of the parasites in the animals.

The leucopoenia observed was mainly due to lymphopenia which was observed in all infected animals, and these cells are believed to decrease in number due to a lymphocidal effect of a lymphotoxin embedded in the membrane of trypanosomes. Lymphocyte death induced by this protein was found to depend on the intervention of a lymphocytic protein tyrosine phosphatase [19] However lymphopenia was reversed following treatment with diminazene aceturate suggesting the destruction of the trypanosomes may have resulted in the loss of the lymphotoxins. This reversal in lymphopenia was not observed in the MEAM treated group and was probably caused by the increase in the lymphotoxins due to a corresponding increase in trypanosomes and the failure of the extract to inhibit multiplication of the trypanosomes.

Monocytopoenia seen in trypanosomosis have been reported by [4],[5]to be due to the immunosuppression caused by the parasite. This report agrees with the findings from this study which showed monocytopenia in the infected groups, but was only reversed in the diminazene aceturate treated group suggesting that the clearance of the trypanosomes from the blood stream led to the return of the mean absolute monocyte count to near pre infection values

V. CONCLUSION

This study shows that the Methanol leaf extract of *Annona muricata* showed trypanocidal activity *In vitro*, however, this activity was not seen *In vivo* at the dose and route of administration used.However, further studies should be done on the plant using a different route of administration such as intramuscularly or intraperitoneally, to assess the trypanocidal efficacy of the leaf of *Annona muricata*

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