

# ***Invitro* and *invivo* antitrypanosomal activities of *Annona muricata* leaf extracts in *Trypanosoma brucei brucei* experimentally infected albino rats**

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## **ABSTRACT**

**Aims:** Trypanosomiasis is an important protozoan disease that affects domestic and wild animals as well as man. It is caused by the tsetse fly-transmitted extracellular hemo-flagellates that belong to the genus, *Trypanosoma*. In East and Southern Africa, Human African Trypanosomiasis (HAT) is caused by *Trypanosoma brucei rhodesiense* while in West and Central Africa, it is caused by *T. b. gambiense*. Animal trypanosomiasis on the other hand is caused by *T. b. brucei*, *T. vivax*, and *T. congolense*. In sub-Saharan Africa, about sixty million people are at risk of infection. This current study evaluates the antitrypanosomal efficacy of extracts of *Annona muricata* leaf in *Trypanosoma brucei brucei* infected albino rats.

**Place and Duration of Study:** Animal parasitology and microbiology research unit, Department of Animal production and Health, Federal University of Technology, Akure, Nigeria, between February and June, 2021.

**Methodology** *Invitro* antitrypanosomal analysis was done in varied concentrations of 2.5mg/ml, 5mg/ml and 10mg/ml using various solvent extracts (ethanolic, ethyl acetate, n-hexane, chloroform and aqueous). Diminazine aceturate and normal saline were used as positive and negative controls respectively. The *invivo* assay was carried out through intraperitoneal administration of graded doses (200, 400 and 600mg/kg) of ethyl acetate and chloroform extracts of the plant for three consecutive days.

**Results:** The n-hexane, chloroform and ethyl acetate extracts yielded high percentage DPPH free radical scavenging activity of 85.52, 80.00 and 78.49% respectively. Decrease in motility of the parasites at different times were observed in all extracts tested *invitro*. These responses were positively concentration-dependent. 10mg/ml concentration of chloroform and ethyl acetate showed complete cessation of parasite motility at 35 and 45 minutes respectively. These two extracts (ethyl acetate and chloroform extracts) which showed the best *invitro* responses, were subjected to *invivo* analysis. Both extracts caused decrease in trypanosome parasitemia and prolongation of mean survival days of the rats to 14.67days as compared with 6.83days in the negative control group. The extracts displayed dose-dependent significant ( $p \leq 0.05$ ) antitrypanosomal activities when compared with the negative and positive controls.

**Conclusion:** The chloroform and ethyl acetate extracts of *Annona muricata* showed a relatively higher antitrypanosomal activity over other solvent extracts used in this study. Further fractionation, purification and isolation should be done to confirm the active components in this plant that is responsible for the antitrypanosomal activities recorded.

*Keywords: Annona muricata, antitrypanosomal activities, solvent extracts, Trypanosoma brucei brucei,*

## 1. INTRODUCTION

Trypanosomiasis, though neglected sub-Saharan African tropical infectious disease, its medical and veterinary importance cannot be overemphasized as it poses threat to 36 countries with the estimated population of 55 million people at risk for the period of 2016-2021. It is caused by a protozoan parasite of the genus *Trypanosoma*, transmitted by the tsetse fly [3]. "Human African Trypanosomiasis (also called sleeping sickness) is caused by *T. b. rhodesiense* and *T. b. gambiense* while *T. brucei brucei* is responsible for African Animal Trypanosomiasis (AAT) (also called Nagana) in West Africa" [1,2]

"African Animal Trypanosomiasis is a parasitic disease which results in serious economic losses in livestock following anemia, lethargy and effects on reproduction with more prominence in cattle. Animals other than livestock, including dogs, can also be affected as untreated cases can be fatal, with very high mortality rate in some outbreaks. In domestic animals, Trypanosomiasis caused by *T. brucei*, *T. equiperdum* and *T. evansi* pose a great socio-economic influence as well as global decreased reproductive efficiency" [4]. "With about 57 million people are at risk of infection [5], Trypanosomiasis can be zoonotic with symptoms such as anemia, fever, weakness and weight loss". "Some proposed interventions to mitigate the disease's effect include the World Health Organization (WHO) programs to eliminate Trypanosomiasis as a public health issue by 2020 and interrupt its transmission by 2030 through renewable public-private partnerships with certain health partners to enable the creation of a WHO-led control and surveillance programme" [5].

Bumduuren *et al.*, [6], suggests *Trypanosoma* species are transmitted not only by tsetse flies (*Glossina* spp.) but by other biting insects. Trypanosomiasis control in Africa has been mainly through chemotherapy and chemoprophylaxis, coupled with vector control programmes, as there has not been any reported effective vaccine against the disease, with diminazene and isometamidium chloride as the main drug control of animal trypanosomiasis in Africa. Their continued use and effectiveness have been threatened by serious limitations, ranging from high cost, long-course of parenteral administration, serious side effects, varied efficacy and emergence of drug resistant trypanosome strains [7]. "The presence of drug resistant trypanosomes has recently risen to alarming proportions [8], hence Osho and Lajide [9] supports the search for new chemical entities which are effective against trypanosomes, safe yet affordable for disease-endemic countries is rational".

"Countless number of secondary molecules that have pharmacological effects present in plants increases their chances as potential sources of new drugs [9,10] which are pharmacologically active and can provide an alternative to chemically synthesized drugs to which many infectious microorganisms have become resistant" [11]. Exploring traditionally appraised medicinal plant for the biological activity has provided humankind with a number of antiprotozoal medications which pastoralists all over the world, have used in old tradition for the treatment of several animal diseases, including trypanosomiasis.

"*Annona muricata* Lin., commonly called soursop, is part of the Annonaceae family. *A. muricata* L. contains various compounds with pharmacological activity. This plant is widely grown in tropical and subtropical areas such as Southeast Asia, South America, and the rainforests of Africa. The plant produces edible fruit all year round and is widely used as a traditional medicine for skin disease, respiratory disease, fever, bacterial infections, diabetes, hypertension, and cancer" [12].

"Validation of medicinal plant for their antitrypanosomal activity will direct the society on the

best approach to employ their indigenous knowledge and at the same time provide hit compounds to feed future regime for antitrypanomal drug development” [13]. This study therefore provides a valid scientific proof for the invitro and invivo therapeutic efficacy of extracts of *Annona muricata* against *Trypanosoma brucei brucei* infected wistar albino rats.

## **2. MATERIAL AND METHODS**

### **2.1 Collection of plant leaves**

Leaves of *Annona muricata* Linn (Annonaceae) were harvested locally during the daytime from their natural habitats in Akure, Ondo State, Nigeria. They were authenticated by the Department of Pharmacognosy, Faculty of pharmacy, Obafemi Awolowo University, Ile ife with voucher number FPI 2400.

### **2.2 Plants extraction**

Fresh leaves of *Annona muricata* were air-dried and crushed with the aid of a mechanical grinder to powdery form. Extraction was done using five different solvents (chloroform, ethanol, hexane, ethyl acetate and distilled water). 200 g dried weight of powdered sample of leaf was soaked in 1.0 liter of individual solvents in order to extract the bioactive components that are present in the plant samples. The sample-solvent combination was agitated at hourly intervals for 72 hours after which mixtures were filtered. The resultant extracts were lyophilized using rotary evaporator (R110) at 40°C to obtain a dry powder extract. Percentage yield of each plant extracts were calculated. The respective extracts obtained were stored in a sealed container and refrigerated until use.

### **2.3 Phytochemical Analysis of Plant Extracts**

Extracts were subjected to quantitative and qualitative phytochemical screening. Methods described by Trease and Evans [14] and Ejikeme *et al.*, [15] were adopted to detect the absence or presence of secondary metabolites such as; saponins, tannins, alkaloids, flavonoids, and phenol in the plant.

### **2.4 DPPH free radical scavenging activities**

Based on the scavenging activities of the stable 1,1-diphenyl 2-picrylhydrazyl (DPPH), the free radical scavenging activity of the extracts were determined adopting the method described by Villano *et al.*, [16].

### **2.5 Trypanosomes and experimental animals**

*Trypanosoma brucei brucei* was obtained from the National Institute of Trypanosomiasis Research, Vom, Jos, Nigeria. Serial passaging of the parasite was done in a donor rat. Parasitaemia of 'donor rat' was estimated using "rapid matching" method. [17].

### **2.6 Experimental rats' acclimatization**

Both male and female healthy adult wistar albino rats of whose weight ranges between 130-180g were procured from the Animal facility of Obafemi Awolowo University Ile Ife, Osun State, Nigeria. They were housed in clean dry cages and fed standard pellets and watered ad libitum. They were kept for seven (7) days to allow them acclimatise and cared for

following the international guidelines for the use and maintenance of experimental animals [18].

### **2.7 Evaluation of invitro antitrypanosomal activity**

Invitro test was carried out in triplicates to assess the motility of trypanosomes using a 96 well microplate. 20 $\mu$ L of blood containing about 16-32 organisms per field were mixed with 5 $\mu$ L of the test substance at concentrations of 2.5, 5, 10mg/mL to produce test concentrations of 0.5, 1, 2.0mg/mL, respectively. The standard trypanocidal drug, diminazene aceturate (DA) and phosphate buffer saline (pH 7.2) were used to serve as treated and untreated controls, respectively. At 37°C, the mixtures were incubated for up to 2hours. Motility of the parasites was checked in intervals of 5 minutes under microscope (X40 objective lens). In summary, about 2 $\mu$ L of test mixtures was placed on microscope slide and covered with cover slips. The parasites were observed for reduced motility or complete cessation of motility. Chloroform and ethyl acetate extracts yielded the best results in this invitro experiment and were further subjected to invivo analysis.

### **2.8 Evaluation of invivo antitrypanosomal activities**

Forty-eight wistar albino rats of both sexes weighing between 130g-180g were used for the invivo phase of this study. The animals were randomly divided into eight groups (I-VIII) consisting 6 rats each. All groups were infected intraperitoneally with 0.2ml of *T. brucei* (at 7.2 antilog) suspension. Groups I-III were treated with chloroform extracts while groups IV-VI were treated with the ethyl acetate extracts of *Annona muricata* at daily doses of 200, 400 and 600mg/kg body weight for three consecutive days intraperitoneally. Groups VII was administered Diminacene aceturate DA at 3.35mg/kg as a single dose intraperitoneally to serve as treated control. 0.3 mL normal saline was administered intraperitoneally to Group VIII for three consecutive days to serve as untreated control. Treatment was initiated on fourth day post inoculation in all groups. Body weight, parasitemia, and rectal temperature were monitored daily for 21days [19]. Surviving rats after day 21 were sacrificed by cervical dislocation.

Parasitemia was monitored through examination of blood from the tail of rats under microscope at  $\times$  40 magnifications using the "Rapid Matching" method as illustrated by Herbert and Lumsden 1979. Parasitemia was monitored on a daily basis until the 21st day from the onset of treatment [20, 21].

Body weight of experimental animals were recorded on the day of parasite inoculation and everyday thereafter for 21days [22].

With the aid of rectal thermometer (Mettler Toledo, Switzerland), rectal temperature was recorded on the day of parasite inoculation and everyday thereafter for 21days [23].

## **3. RESULTS**

### **3.1 Yield**

Ethanol gave the highest yield followed by n-hexane, chloroform, aqueous and ethyl acetate in that order.

**Table 1: Percentage yield with the different solvents of extraction**

S/N	EXTRACTS	% YIELD
1	EEAM	13.61
2	EAAM	18.11
3	NHAM	15.31
4	AEAM	13.59
5	CEAM	16.30

EEAM: Ethanolic extract of *A. muricata*  
EAAM: Ethyl acetate extract of *A. muricata*  
NHAM: N- hexane extract of *A. muricata*  
AEAM: Aqueous extract of *A. muricata*  
CEAM: Chloroform extract of *A. muricata*

### 3.2 DPPH free radical scavenging activities of extracts of *Annona muricata* leaves

N-Hexane extract gave the highest DPPH free radical scavenging percentage followed by chloroform, ethyl acetate, ethanolic and aqueous extracts in that order

**Table 2: Percentage DPPH free radical scavenging activities with the different solvents of extraction**

Sample	DPPH (%)
EEAM	77.38
EAAM	78.49
NHAM	85.52
AEAM	55.56
CEAM	80.00

EEAM: Ethanolic extract of *A. muricata*  
EAAM: Ethyl acetate extract of *A. muricata*  
NHAM: N- hexane extract of *A. muricata*  
AEAM: Aqueous extract of *A. muricata*  
CEAM: Chloroform extract of *A. muricata*

### 3.3 Phytochemical screening

Phytochemical screening revealed the presence of saponins, tannins, phenol, flavonoids and alkaloids in varying quantities with the different extraction solvents used (Table 3 and 4).

**Table 3: Qualitative phytochemical analysis of extracts of *Annona muricata* leaves**

Sample	Alkaloids	Saponin	Tannin	Flavonoid	Terpenoids	Steroid
EEAM	+	+	+	+	-	+

EAAM	++	++	+	+	+	+
NHAM	++	++	+	+	+	+
AEAM	+	++	±	+	±	+
CEAM	++	+	+	+	+	+

Foot note:

+: Present      - : Absent      +: Slightly present      ++: Highly present

**Table 4: Quantitative phytochemical analysis of extracts of *Annona muricata* leaves**

Sample	Alkaloids %	Saponin %	Flavonoids mg/100g	Phenol mg/100g	Tannin mg/100g
EEAM	6.98	9.59	7.64	3.43	5.00
EAAM	27.77	16.40	3.14	3.78	5.08
NHAM	21.03	26.49	3.86	3.68	4.63
AEAM	3.50	32.08	6.64	0.07	0.08
CEAM	23.16	7.89	2.50	3.11	3.68

### 3.4 *In vitro* antitrypanosomal activity

Chloroform and ethyl acetate extracts of *A. muricata* leaves ceased motility of the trypanosomes within 40 minutes at 10mg/ml concentrations (Table 5).

**Table 5: Effect of different extracts of *Annona muricata* on motility cessation time of *Trypanosoma brucei brucei* *in vitro***

Conc.	Time (min) After Which Motility Ceased						DA	NC
	EEAM	EAAM	NHAM	AEAM	CEAM			
10mg/ml	75	40	65	95	30	15	NE	
5mg/ml	80	55	70	105	35	20	NE	
2.5mg/ml	95	65	80	115	60	35	NE	

### 3.5 Effect of treatment on parasitemia

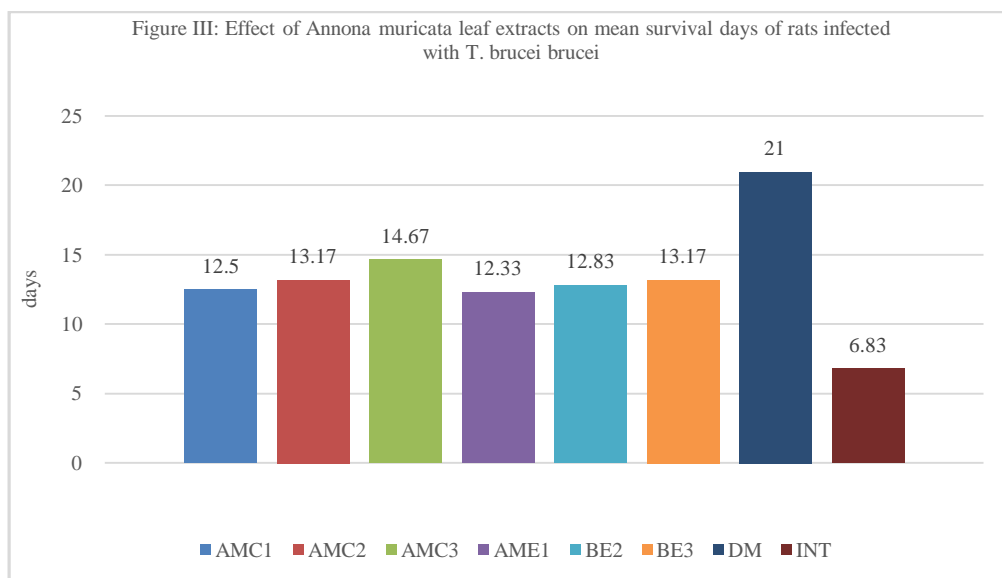
Parasitemia level assessed at day 5 post-treatment in all experimental animals for the different groups showed that all doses of the extract including the standard drug suppressed parasitemia ( $p < 0.05$ ). The best result was obtained with chloroform extract of *A. muricata* at 400mg/kg on day 11 (Figure 1).



**Figure 2: Effect of *Annona muricata* leaf extracts on weight of *T. brucei brucei* infected rats**

### **3.7 Effect of treatment on mean survival days**

The average survival days (post treatment) of infected rats were increased to 13.33 and 14 days in groups treated with 600mg/kg of ethyl acetate and chloroform extracts respectively compared with 6.83 days in negative control group (Figure 3).



**Figure 3: Effect of *A. muricata* leaf extracts on mean survival days of rats infected with *T. brucei brucei***

### **3.8 Effects of treatment on rectal temperature**

The rectal temperatures of the rats fluctuated throughout the duration of the experiment independent of the environmental temperatures. There is no observable significant difference nor pattern.

## **4. DISCUSSION**

The affinity of chemical substances for the different solvents of extraction is responsible for variations observed in yield of extracts. Solubility of compounds tend to be higher in polar solvents such as methanol in comparison with non-polar solvent such as chloroform [24]. Nguyen et al., [25] also reported variation in yield of *Annona muricata* seeds using distilled water, ethanol, ethyl acetate and chloroform, where chloroform gave the highest yield.

“Different roles of phytochemicals have been reported ranging from plants’ protection from harmful insects, ultraviolet (UV) rays, microbes and extreme temperatures. They also play important roles in man and animals. Most phytochemicals have been observed to display antioxidant activities as they help in mopping up free radicals and also protect against diseases that manifest as a result of free roaming reactive oxygen species (ROS)” [26, 27, 28]. “Phytochemicals have been documented to help prevent and ameliorate diseases such as diabetes, cancer, hyperlipidaemia, Alzheimer, cardiovascular diseases, liver toxicity, cataract, age related function decline, stroke and others. Various phytochemicals that have

been isolated from plants include alkaloids, glycosides, saponins, flavonoids, kaempferol, phytol, gallic acid, kolaviron and essential oils" [29].

The qualitative and quantitative phytochemicals obtained from both plants differ with the various solvent used for the extraction. Significant pharmacological functions such as; antimalarial, anticancer, analgesic, and anti-hyperglycemic and antibacterial functions have been reported for alkaloids [30]. Steroids are used to relief inflammatory conditions while terpenoids have antioxidant activities. Saponins have been evidenced to possess anticoagulant, anti-carcinogenic, hypoglycemic, immunomodulator, neuroprotective, anti-inflammatory and antioxidant potentials. Glycosides have antimicrobial and anticancer activities. [31].

Traditional uses of *Annona muricata* in the treatment of various diseases can be credited to the availability of these constituents in its leaf

Antitrypanosomal and therapeutic properties of some medicinal plants have been associated with the significant levels of various classes of phytochemicals compounds or secondary metabolites present in them. The leaf extract of *Annona muricata* contains alkaloids, saponins, tannins, phlobatannins, and flavonoids. These phytochemicals have been reported earlier to be present in the seeds of *A. muricata* [32].

The decrease in body weight of experimental rats over a period might be due to anorexia which is one of the clinical features of trypanosomosis. This can further be described by the inverse relationship between level of parasitaemia and weight loss of gain in the treatment groups; a relationship that is positively dose dependent.

Chloroform extracts of *A. muricata* exhibited higher antitrypanosomal activities than its ethyl acetate extract both invitro and invivo. This fact may suggest the concentration of the plant's pharmacological active ingredients responsible for its antitrypanosomal activities by chloroform solvent to a large extent. Diminazene aceturate treatment group displayed the highest antitrypanosomal activities in the infected rats because the parasite has been tested sensitive to diminazene being a standard antitrypanosomal drug. The effectiveness of diminazene aceturate at very low dosages is because its purity when compared with the crude extracts of *Annona muricata* that were used in this study in which relapse was recorded.

## 5. CONCLUSION

The chloroform and ethyl acetate extracts showed a relatively higher antitrypanosomal activity over other solvent extracts used in this study.

The extracts of *A. muricata*, in this study can be beneficial to human and animal health in the treatment of trypanosomiasis.

This study provides a lead for researchers to uncover the specific bioactive components of *Annona muricata* that exerts the antitrypanosomal efficacy.

## ETHICAL APPROVAL

All of the animals used in this study received humane care according to the criteria outlined in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy Science and published by the National Institute of Health (USA). The ethic regulations have been followed in accordance with national and institutional guidelines for the protection of animals' welfare during experiments.

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