Antitrypanosomalefficacy of*Annonamuricata*leafextractsin *Trypanosomabruceibrucei*experimentally infected rats

ABSTRACT

Aims: Trypanosomiasis is an important protozoan disease that affects domestic animals and 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 Ttrypanosomiasis (HAT) is caused by ~~the tsetse fly transmitted hemo flagellates~~ *Trypanosomabruceirhodesiense* 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*. The incidence of sleeping sickness as a major health problem in many African countries has been on the rise in recent years. 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 Trypanosomabruceibrucei 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 usingvarious solvent extracts (ethanolic, ethyl acetate, n-hexane, chloroform and aqueous).Diminazineaceturate 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 activitieswhen compared with the negative and positive controls.

Conclusion: The chloroform and ethyl acetate extracts of *Annonamuricata* showed a relatively higher antitrypanosomalactivity 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, Trypanosomabruceibrucei,

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1. INTRODUCTION

Trypanosomiasis, though neglected sub-Sahara African tropical infectious disease, its medical and veterinary importance cannot be overemphasized. It is caused by a protozoan parasite of the genus Trypanosoma, transmitted by the tsetse fly [3] within the distributional limits.Trypanosoma species *T. bruceibrucei* responsible for African Animal Trypanosomiasis (AAT) called Nagana in West Africa, *T. b. rhodesiense* and *T. b. gambiense* cause Human African Trypanosomiasis (HAT) or sleeping sickness [1, 2]

African animal trypanosomiasisis 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 [5].

Bumduurene*t al.,* [6], suggests Trypanosoma species are transmitted not only by tsetse flies (Glossina spp.) but by other biting insects. Trypanosomosis 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 trypanosomosis 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 trypanosomosis.
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 antitrypanomal 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 *Trypanosomabruceibrucei* infected wistar albino rats.

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Comment [AE10]: Different statements for AAT and HAT would make the message more clear. Start with HAT then talk about AAT.

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Much as I can tell that you are targeting treatment for AAT, it is possible that these molecules can also make it to the pipeline for human use.
Chemotherapy for HAT has similar problems of limited drugs which are very toxic.

I suggest therefore you extend this problem statement to the human side because you talk about possible benefits to human health in your conclusion

2. MATERIAL AND METHODS

2.1 *Collection of plant leaves*

Leaves of *Annonamuricata* 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~~Pharmacognosis~~, Faculty of pharmacy, Obafemi Awolowo University, Ile ife with voucher number FPI 2400.

2.2 *Plants extraction*

Fresh leaves of *Annonamuricata* 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-dipheny 1-2-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*

Trypanosomabruceibrucei was obtained from the National Institute of Trypanosomosis 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' acclamatization*

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

2.7*Evaluation of invitroantitrypanosomal activity*

Invitro test was carried out in triplicates to assess the motility of trypanosomes using a 96 well microplate. 20µL of blood containing about 16-32 organisms per field were mixed with 5µL 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, diminazeneaceturate (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 2µL 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 invivoantitrypanosomal 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 *Annonamuricata* at daily doses of 200, 400 and 600mg/kg body weight for three consecutive days intraperitoneally. Groups VII was administered Diminaceneaceturate DA at 3.35mg/kg as a single dose intrapenitoreally 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].

Parasitemia was monitored through examination of blood from the tail of rats under microscope at × 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 I: 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 II: Percentage DPPH free radical scavenging activitieswith 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 III and IV).

Table III: Qualitative phytochemical analysis of extracts of *Annonamuricata* leaves

Sample	Alkaloids	Saponin	Tannin	Flavonoid	Terpenoids	Steroid
EEAM	+	+	+	+	-	+
EAAM	++	++	+	+	+	+
NHAM	++	++	+	+	+	+
AEAM	+	++	±	+	±	+
CEAM	++	+	+	+	+	+

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

Table IV: Quantitative phytochemical analysis of extracts of *Annonamuricata* 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*Invitroantitrypanosomal activity*

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

Table V: Effect of different extracts of *Annonamuricata* on motility ceasation time of *Trypanosomabrucei brucei* invitro

Conc.	Time (min) After Which Motility Ceased						
	EEAM	EAAM	NHAM	AEAM	CEAM	DA	NC
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 I).

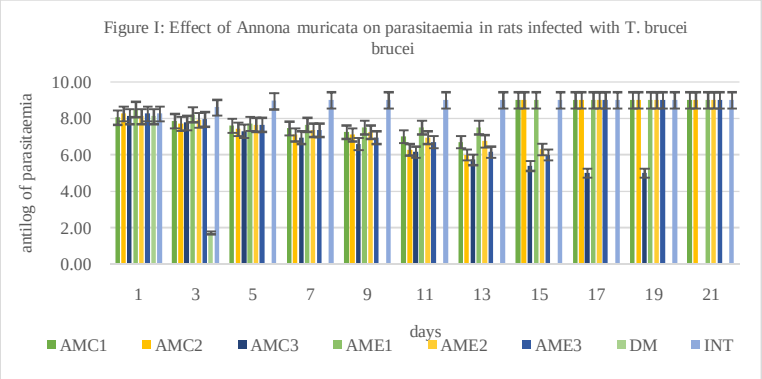

Figure I: Effect of A. muricata on parasitaemia in rats infected with *T. bruceibrucei*
Notes
AMC1: Chloroform extract of *A. muricata* at 200mg/kg
AMC2: Chloroform extract of *A. muricata* at 400mg/kg
AMC3: Chloroform extract of *A. muricata* at 600mg/kg
AME1: Ethyl acetate extract of *A. muricata* at 200mg/kg
AME2: Ethyl acetate extract of *A. muricata* at 400mg/kg
AME3: Ethyl acetate extract of *A. muricata* at 600mg/kg
DM :Diminazeneaceturate at 3.35mg/kg
INT: Infected not treated

3.6*Effect of treatment on body weight*

There iwas statistically significant ($p<0.05$) body weight changes in 600mg/kg and DA 3.35mg/kg treated groups compared with 200mg/kg, 400mg/kg, treatment groups with both chloroform and ethyl acetate extracts from day 8 to day 14 post-treatment initiation and with untreated control on day 5 post-treatment (Figure II).

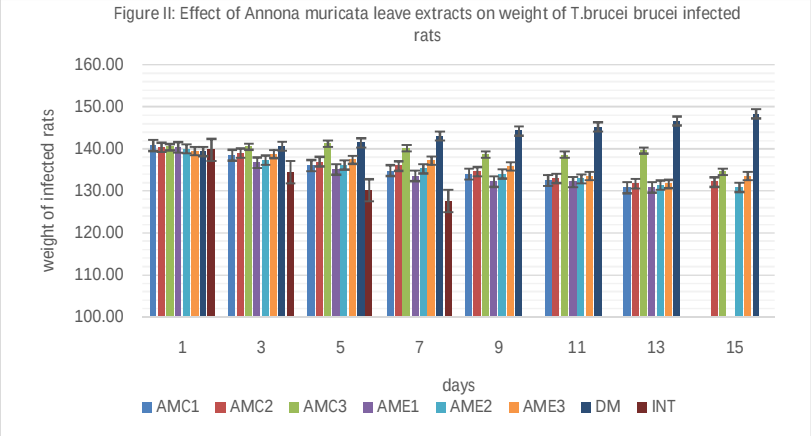

Figure II: Effect of Annona muricata leave extracts on weight of T. bruceibrucei infected rats

3.7*Effect of treatment on mean survival days*

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

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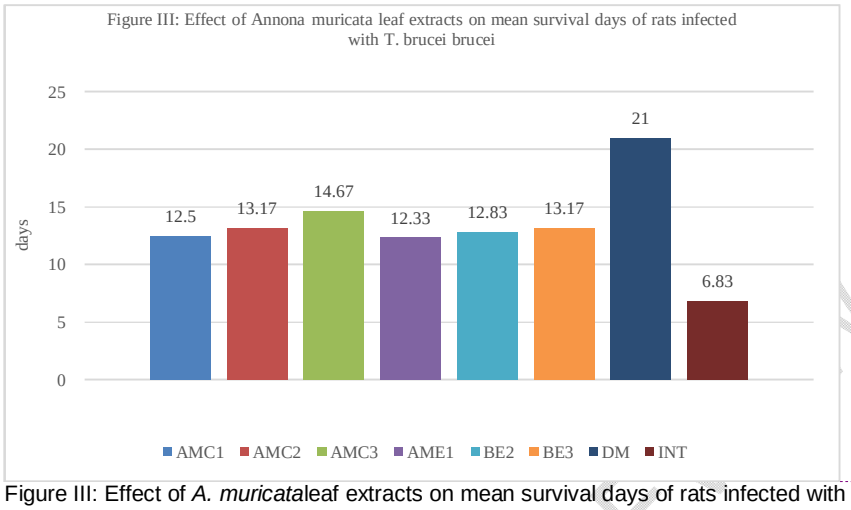

Figure III: Effect of *A. muricata*leaf extracts on mean survival days of rats infected with *T. bruceibrucei*

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 composition of 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 ~~comparism~~ 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 antioxidantactivities 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

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anticoagulant, anti-carcinogenic, hypoglycemic, immunomodulator, neuroprotective, anti-inflammatory and antioxidant potentials. Glycosides have antimicrobial and anticancer activities. [31].
Traditional uses of *Annonamuricata* 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. muricate [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. Diminazeneaceturate 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 diminazeneaceturate at very low dosages is because its purity when compared with the crude extracts of Annona muricate that were used in this study in which relapse was recorded.

5. CONCLUSION

The plant antitrypanosomal activities may be associated to the presence of active secondary metabolites in the leaf and this affirms its use in Nigerian local communities towards the treatment of parasitic infection. The chloroform and ethyl acetate extracts showed a relatively higher 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 trypanosomosis.

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

CONSENT

Not applicable

ETHICAL APPROVAL

Not applicable

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Comment [AE20]: I cant seem to find the basis for saying secondary metabolites are responsible. You did not do pharmacokinetics or pharmacodinamics on a specific primary compound.

Comment [AE21]: I dont think this statement is necessary in the conclusion. It is loaded with speculation. A lay person reading this will take it that you are further afirming its use.

I think your message in the conclusion needs to be simplified to your findings that extracts of *A. Muricata*have antitrypanosomal activity.

Comment [AE22]: I dont agree with this. The authors used 48 mice. Various countriees and research institutions have guidelines/ethics bodies that review work with animals.
If the work had stopped on invitro tests, ethics approval may not have been required.

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