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herba polonica
From Botanical to Medical Research
Vol. 61 No. 2 2015
DOI: 10.1515/hepo-2015-0015

EXPERIMENTAL PAPER

In vitro and *in vivo* evaluation of antitrypanosomal activity of *Annona muricata* stem bark extracts

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Summary

The control of trypanosomosis in animals and humans based on chemotherapy is limited and not ideal, since the agents used are associated with severe side effects, and emergence of relapse and drug resistant parasites. The need for the development of new, cheap and safe compounds stimulated this study. Three concentrations (211, 21.1 and 2.11 mg per ml) of chloroform stem bark extract of *Annona muricata* were screened for trypanocidal activity against *Trypanosoma brucei brucei in vitro.* Also, two doses (200 mg per kg and 100 mg per kg) of the extract were evaluated for trypanocidal activity in rats infected with the parasite. Haematological parameters were determined on day 1 post infection and on days 1, 6 and 30-post extract treatment. The extracts inhibited parasite motility and totally eliminated the organisms at the concentrations used *in vitro.* The extract also showed promising *in vivo* trypanocidal activity. The observed *in vitro* and *in vivo* trypanocidal activities may be due to the presence of bioactive compounds present in the extracts as seen in this study. The extract also improved the observed decreases in haematological parameters of the treated rats, which may be due to their ability to decrease parasite load. The observed oral LD_{50} of 1,725.05 mg per kg of the chloroform *A. muricata* extract using up and down method is an indication of very low toxicity, implying that the extract could be administered with some degree of safety.

Key words: *Annona muricata, trypanosomosis, extract, treatment, Trypanosoma brucei brucei*

INTRODUCTION

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 African trypanosomosis in humans, while five drugs (diminazene, isometamidium, homidium bromide and chloride and quinapyramine compounds) are used for the treatment of animal trypanosomosis. The drugs used for the treatment of trypanosomosis, both in animals and man are beset with challenges of severe toxicity and increasing incidence of trypanosome resistance and relapse [1-3]. This situation suggests the need to develop newer, easily available and safer trypanocides [4, 5], by exploring efficacious chemotherapeutic agents from locally available medicinal plants.

Annona muricata is a member of the family of custard apple trees called *Annonaceae*. It produces a fruit called soursop due to its slight acidic taste when ripe. *Annona muricata* trees also called graviola (common name) are native to the Caribbean and Central America but now are widely cultivated in tropical climates throughout the world [6]. Available scientific evidence revealed that *A. muricata* plant has a long history of natural medicinal uses. All parts of the plant are used in natural medicine in the tropics [6-8]. Some of its uses in natural medicine such as hypotensive, antispasmodic, anticonvulsant, vasodilator and smooth muscle relaxant activities have been validated by scientific research in animal studies [6, 9, 10].

Graviola seeds were observed to have anti-parasitic properties and the leaf extract showed activity against malaria parasites [8]. It also has anti-cancer properties [11]. Phytochemical screening revealed that *A. muricata* stem-bark extract is rich in flavonoids and tannins amongst others. Examples of phytochemicals isolated from *A. muricata* include annonaceous acetogenins, annocatalin, annohexocin, annomonicin, muricapentocin, xylomaticin etc. The aim of this study is to evaluate the efficacy of *A. muricata* in the treatment of trypanosomal infection. The need for a locally produced and potent antitrypanosomal agent and the fact that *A. muricata* has been earlier used to eliminate malaria parasites stimulated this present study.

MATERIAL AND METHODS

Plant material

Fresh samples of *Annona muricata* stem bark and leaves were collected from Mmoguluwa's compound, Ifite village, Nwafia, Anambra State, Nigeria. The leaves were identified and authenticated at the Herbarium unit of the Department of Botany, University of Nigeria, Nsukka.

Plant extraction

The water, methanol and chloroform extracts of the plant were prepared according to the method described by Vieira *et al*. [12] and modified by Akanji *et al*. [13]. Fresh samples of *A. muricata* stem bark were air dried and ground. The ground sample weighing 600 g was separated into three equal parts (200 g each), and macerated respectively in distilled water, methanol and chloroform for 48 hours with occasional stirring or agitation on a mechanical shaker. The extracts were filtered through Whatman No 1 filter paper. Each extract was evaporated to dryness *in vacuo*. The residues were collected and kept in separate capped bottles at 4°C until used. On the day of experiment a fresh stock solution was prepared from the residue.

Animals

Sprague Dawley Albino rats were obtained from the Department of Veterinary Physiology, Pharmacology and Biochemistry animals unit, University of Agriculture, Makurdi, Nigeria. They were housed in standard rat cages, in the Department of Veterinary Physiology, Pharmacology and Biochemistry Laboratory and were allowed two weeks to acclimatize before the start of the experiments. All animals were handled according to the international guiding principles for Biomedical Research involving Animals [14], as permitted by University of Agriculture, Makurdi ethical committee concerning the use of laboratory animals given the permit number (P/No: 2014 014).

Acute toxicity study

The up and down procedure of Dixon [15] was adopted. The calculation of median lethal dose was used according to the method adopted by Saganuwan *et al*. [16].

Phytochemical analysis

The crude extracts of aqueous, methanol and chloroform of the stem bark of *Annona muricata* were subjected to qualitative chemical screening for active chemical constituents [17, 18].

Test organism

Trypanosoma brucei brucei (*T. b. brucei*) was obtained from stabilates maintained at the Nigerian Institute for Trypanosomiasis Research (NITR), Vom. Plateau State.

The parasite was maintained in the Laboratory by continuous passage in rats until required. Passage was considered necessary when parasitaemia was in the range of 16-32 parasites per field [19] usually 3-5 days post infection.

Determination of parasitaemia

Parasitaemia was monitored in blood obtained from the tail vein presterilized with methylated spirit. The number of parasites was determined microscopically at x 400 magnification using the "Rapid Matching" method of Herbert and Lumsden [19]. The blood was appropriately diluted with buffered phosphate saline (BPS, pH 7.2) solution.

In vitro test for trypanosome activity

Assessment of *in vitro* trypanocidal activity of the chloroform extract was performed in triplicates in 96 well microliter plates (Flow Laboratories Inc, McLean, Virginia 22101, USA). Twenty microliter (20 μl) of blood containing about 20-25 parasites per field was mixed with 10 μl of extract solution of 422, 42.2 and 4.22 mg per ml to produce effective test concentrations of 211, 21.1 and 2.11 mg per ml, respectively. To ensure that the effect monitored was that of the extract alone, a set of control was introduced which contained the parasite suspended in BPS only. For reference, tests were also performed with concentrations of Tryponil^R (445 mg diminazene diaceturate x 555 mg phenazone per g, Horster way 26A, 5811 AC centenary, Holland) a commercial trypanocide drug.

After 5 min of incubation in a water bath maintained at 37^{o}C, 2 μl of test mixtures were placed on separate microscope slide and covered with cover slips and the parasites observed every 10 min for a total of 60 min. Cessation or drop in motility of the parasites in extract treated blood compared to that of parasite loaded control blood without extract was taken as a measure of trypanocidal activity.

In vivo efficacy of *Annona muricata* stem bark in *Trypanosoma* infected rats

Twenty-five rats of both sexes (136–194 g) divided into five groups (A-E) of five rats each, kept in clean rat cages, and fed standard diet and water *ad libitum* were used.

Rats in groups B to E were infected intraperitoneally with infected blood in BPS containing $1x10^3$ trypanosomes. Group A served as the control and received BPS only.

Group B was infected untreated control while group C was treated with deminazene aceturate (3.5 mg per kg) once, intraperitoneally. Groups D and E were treated orally with chloroform extract of *A. muricata* at 100 and 200 mg per kg doses respectively for 6 days. The treatments commenced 6 days post infection when parasitaemia was well established based on wet mount and blood film examination. Active infections with trypanosomes were detected by examination of smears made from the tail vein every three days. Blood samples collected by cardiac puncture into EDTA bottles on days 1, 6, 12 and 36 were used for the analysis of the following, haematological parameters: red blood cells counts (RBC), packed cells volume (PCV) hemoglobin concentration (Hb), and white blood cells (WBC) counts [20].

Statistical analysis

The data obtained from the study were summarized as mean ± standard error using Microsoft excel. Two way analysis of variance (ANOVA) was used for comparisons. *P*-values greater than 0.05 were considered significant.

RESULTS

Acute toxicity: the oral median lethal dose (LD_{50}) of *A. muricata* in rats was calculated to be 1,723.05 mg per kg. One rat was administered with a limited dose of 3000 mg/kg died within 48 hours of dosing with the toxicity signs of restlessness, dyspnea, anorexia, starry hair coat and sternal recumbence.

Phytochemical analysis

The phytochemical screening of the crude aqueous, methanol and chloroform extraction is shown in table 1. The results indicate high presence of flavonoids in the chloroform extract and moderate and low presence of flavonoids in the methanol and aqueous extracts, respectively.

Phlobatanins and saponins were found only in the chloroform extract. Steroids and tannins were moderately present in *A. muricata* chloroform extract compared to the methanol extract with low concentrations. Carbohydrates and cardiac glycosides were present in low concentrations in the three extracts. The concentration of alkaloid was more in the chloroform extract than the methanol and aqueous extracts.

Table 1.

Phytochemistry of the aqueous, methanol and chloroform extracts of *Annona muricata* stem bark

Extracts				
Chemical constituent	Type of test	Aqueous	Methanol	Chloroform
Alkaloid	Wagner	–	–	–
	Dragendorff's	+	+	++
Flavonoids	Shinoda	–	–	–
	Ferric chloride	+	++	+++
	Lead acetate	+	++	+++
Cardiac glycoside	Keuar-Kiliani	+	+	+
Phlobatanins	HCl	–	–	++
Saponins	Frothing	–	–	++
Steroids	Liebermann's	–	+	++
Tannins	Bruemer's	+	+	++
Terpenoids	Liebermann's	–	+	–
Carbohydrate	Molisch's	+	+	+

\+ – low concentration
++ – moderate concentration
+++ – high concentration
– – absent

In vitro trypanocidal efficacy of *A. muricata*: chloroform stem bark extract of *A. muricata* at the concentrations used in this study eliminated trypanosomal motility within 10 min. This effect was observed for diminazene aceturate at 30 min, of incubation. The control had little or no effect on the trypanosome parasites (tab. 2). At 60 min of incubation, the trypanosome parasites were still present but sluggish in the control group.

Table 2.

In vitro efficacy of chloroform extract of *Annona muricata* stem bark on *Trypanosoma brucei brucei*

	Extract/drug concentrations [mg/ml]				
Time (minute)	Control	EX 2.11	EX 21.1	EX 211.0	DM 6.6
0	++++	++++	++++	++++	++++
10	++++	X	X	X	+++
20	++++	X	X	X	++
30	++++	X	X	X	X
40	++++	X	X	X	X
50	++++	X	X	X	X
60	S	X	X	X	X

++++ – presence of 4 parasites/ field
+++ – 3 parasites/field
++ – 2 parasites/field
X – absence of parasites
S – parasite present but sluggish

In vivo efficacy of *A. muricata*: the comparative trypanocidal efficacy of *A. muricata* and diminazene aceturate in *T. b. brucei* infected rats are shown in table 3. The infected rats developed teeming parasitaemia within 6 days of inoculation with the parasite. There was no detectable level of *T. b. brucei* in the blood of rats 3 days after treatment with diminazene aceturate. The same was observed in 100 and 200 mg per kg doses of *A. muricata* extract in treated rats, in 6 days following extract administration.

Table 3.

Comparative trypanocidal efficacy of *Annona muricata* chloroform stem bark extracts and diminazene aceturate in *T. b. brucei* infected rats

Treatment	Parastaemia												
Days after infection	1	3	6	9	12	15	18	21	24	27	30	33	36
Uninfected control	0	0	0	0	0	0	0	0	0	0	0	0	0
Infected untreated control	0/5	1/5	5/5	5/5	5/5	5/5	4/4	4/4	1/1	0	0	0	0
Diminazene aceturate (3.5 mg/kg)	0/5	2/5	5/5	0/5	0/5	0/5	0/5	0/5	2/5	2/5	1/4	1/4	0/3
Annona muricata (100 mg/kg)	0/5	2/5	5/5	2/5	0/5	0/5	2/5	2/5	2/5	2/5	0/3	0/3	0/3
Annona muricata (200 mg/kg)	0/5	3/5	5/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

The infected untreated rats exhibited progressive parasitaemia that resulted in early deaths. Two animals, each treated with 100 mg per kg dose of *A. muricata* and diminazene aceturate, relapsed on days 18 and 24 post infection (i.e. days 12 and 18 post commencement of treatment) and these animals died before the termination of study.

Table 4 shows the mean red blood cells count of rats infected with *T. b. brucei* and subsequently treated with diminazene aceturate and extracts of *A. muricata*. The RBC pre-infection values of the treated rats were not significantly ($p>0.05$) different from those of the control and infected untreated groups. Following infection, the RBC counts of the uninfected control increased, while those of the infected groups decreased significantly ($p<0.05$). However, following treatments, the RBC counts of diminazene aceturate and *A. muricata* treated groups improved at 24 days of commencement of treatments, their values were similar to those of the control. The RBC counts of the infected untreated control were significantly ($p<0.05$) very low compared to the preinfection values, and all the animals in this group died prior to the termination of the study.

The mean packed cells volume (tab. 5) of the infected rats seems to follow the same trend as the RBC counts. Treatments did significantly ($p<0.05$) improve the PCV values. The mean Hb concentration (tab. 6) decreased from the day 1 infection values of 10.27 ± 0.51, 10.73 ± 0.32, 10.73 ± 0.32 and 11.67 ± 1.12 g per dl for infected untreated, diminazene aceturate, *A. muricata* 100 mg per kg

and 200 mg per kg respectively to 6.78±1.83, 9.00±0.67, 5.00±1.71 and 9.22±0.62 g per dl, 6 days post commencement of extract treatment (i.e. 12 days post infection). The hemoglobin values of the treated groups, however, improved and by day 36 of infection (30 days after commencement of treatment). All the infected rats showed leucocytosis, which improved with treatment (tab. 7).

Table 4.

The mean* red blood cells count of rats infected with *T. b. brucei* and treated** with chloroform stem bark extract of *Annona muricata*

Treatment	Red blood cells count [x10^{12}/L]			
Days post-infection	1	6	12	36
Control	4.96±0.27	5.92±1.00^{c}	5.17±0.47^{c}	5.20±0.29^{c} p
Infected untreated	5.10±0.25	4.40±0.28^{b,d}	3.27±0.92^{a,d}	D
Diminazene aceturate (3.5 mg/kg)	5.36±0.14	3.80±0.29^{b,e}	4.53±0.33^{a,e}	5.11±0.41***
Annona muricata (100 mg/kg)	5.34±0.15	4.88±0.19^{b,d}	2.50±0.82^{a,f}	5.06±0.32***
Annona muricata (200 mg/kg)	5.82±0.56	4.42±0.18^{a,d}	4.63±0.32^{a,d}	5.26±0.51

* – mean ±SEM based on 5 observations
** – treatment commenced 6 days post infection
*** – two animals each lost from these groups
D – all the animals in this group died
a, b – values with different superscripts on the same row are statistically significant ($p<0.05$)
c, d, e, f – values on the same column with different superscripts are statistically significant ($p<0.05$)

Table 5.

The mean* packed cells volume of *T. b. brucei* infected rats treated** with varying doses of chloroform stem bark extract of *Annona muricata*

Treatment	Packed cells volume [%]			
Days post-infection	1	6	12	36
Control	31.25±0.85	37.40±2.94^{a,c}	40.67±1.45^{a,c}	42.10±1.60^{a,c}
Infected untreated	30.80±1.53	24.00±1.38^{a,d}	20.33±5.49^{a,d}	D
Diminazene aceturate (3.5 mg/kg)	32.20±0.86	29.20±1.62^{b,e}	27.00±2.00^{b,e}	33.20±1.02***d
Annona muricata (100 mg/kg)	32.20±0.97	31.20±0.86^{e}	15.00±5.13^{a,c}	31.60±1.12***d
Annona muricata (200 mg/kg)	35.00±3.35	33.20±0.66^{e}	27.67±1.86^{a,e}	40.10±1.39^{c}

* – mean ±SEM based on 5 observations
** – treatment commenced 6 days post infection
*** – two rats each lost from these groups
D – all the animals in this group died
a, b – values with different superscripts on the same row are statistically significant ($p<0.05$)
c, d, e, f – values on the same column with different superscripts are statistically significant ($p<0.05$)

Table 6.

The mean* hemoglobin values of *T. b. brucei* infected rats treated** with different doses of chloroform stem bark extracts of *Annona muricata*

Treatment	Hemoglobin concentration [g/dl]			
Days post-infection	1	6	12	36
Control	9.93±0.53	12.47±0.98[a]	13.56±0.48[a,b]	14.3±0.12[a,b]
Infected untreated	10.27±0.51	11.34±0.46	6.78±1.83[a]	D
Diminazene aceturate (3.5 mg/kg)	10.73±0.32	9.73±0.54[a]	9.00±0.67[a,c]	12.51±0.20***
Annona muricata (100 mg/kg)	10.73±0.32	10.40±0.29	5.00±1.71[a]	11.90±.0.42***
Annona muricata (200 mg/kg)	11.67±1.12	11.07±0.22	9.22±0.62[a,c]	12.81±0.25

* – mean ±SEM based on 5 observations
** – treatment commenced 6 days post infection
*** – two rats each lost from these groups
D – all the animals in this group died
a – values with different superscripts on the same row are statistically significant ($p<0.05$)
b, c – values on the same column with different superscripts are statistically significant ($p<0.05$)

Table 7.

The mean* white blood cells counts of *T. b. brucei* infected rats treated** with chloroform stem bark extracts of *Annona muricata*

Treatment	White blood cells count [x$10^{9/l}$]			
Days post-infection	1	6	12	36
Control	5.68±0.68	4.74±0.90	5.43±0.59	5.59±0.25
Infected untreated	5.50±0.70	9.54±0.93a,b	8.70±32a,b	D
Diminazene aceturate (3.5 mg/kg)	6.54±1.09	9.24±1.54a,b	4.37±0.20	5.54±0.35***
Annona muricata (100 mg/kg)	4.84±0.58	7.34±0.68[a,c]	4.67±0.35	5.28±0.31***
Annona muricata (200 mg/kg)	4.66±0.44	7.44±0.62[a,c]	5.13±0.20	5.43±0.28

* – mean ±SEM based on 5 observations
** – treatment commenced 6 days post infection
*** – two rats each lost from these groups
D – all the animals in this group died
a – values with different superscripts on the same row are statistically significant ($p<0.05$)
b, c – values on the same column with different superscripts are statistically significant ($p<0.05$)

DISCUSSION

The oral LD_{50} of the chloroform extract of *A. muricata* stem bark extract of 1,725.05 mg per kg observed in this study, was an indication of low toxicity of the extract. Clarke and Clarke [21] were of the opinion that substances who's LD_{50} are above 1000 mg per kg are of low toxicity. This is an indication that the extract could be administered with some degree of safety especially through oral route,

where absorption may not be complete due to inherent factors limiting absorption in the gastrointestinal tract [27].

The phytochemical study on *A. muricata* stem bark extracts revealed a strong presence of useful classes of chemical compounds such as flavonoids, alkaloids, steroids, tannins, saponins and phlobatanins in the chloroform extract compared to the aqueous and methanol extracts. These classes of chemical compounds have been known to exert pharmacological and antagonistic effects, and some are capable of protecting the active ingredients in herbs from decomposing either chemically or physiologically [23, 24].

Annona muricata showed in this study promising *in vitro* trypanocidal efficacy (tab. 2). Earlier reports [25-27] have clearly indicated that plants of different families could possess potent trypanocidal activity. In the present study, the *A. muricata* extract appeared more effective *in vitro* compared to diminazene aceturate in inhibiting the parasites motility and completely eliminated the parasite within 10 min while diminazene aceturate achieved the same effect at 30 min. There was a consistent suppression of parasitaemia with prolonged survival time of treated rats in the *in vivo* study by 200 mg per kg of *A. muricata* extract. This effect was superior to that of dimiazene aceturate and *A. muricata* (100 mg per kg) treated groups. The outstanding *in vivo* and *in vitro* activity of *A. muricata* extract may be attributed to the presence of alkaloids, flavonoids, tannins and steroids present in the extract. *A. muricata* is known to be rich in flavonoids and tannins amongst others [8, 10, 28-30]. Furthermore, natural products with trypanocidal activity and belonging to a variety of phytochemical classes have been identified [31-33]. The prolongation of lives of rats treated with 200 mg per kg extract dose may also be associated to its ability to improve the observed decreases in their haematological parameters, which may be due to its ability to decrease parasites load. Early deaths observed in the infected untreated group in this study may result from massive parasitaemia, which could have induced haemolytic anaemia in those animals. Anaemia is a consistent finding in *T. b. brucei* infection [5, 34].

CONCLUSION

Annona muricata stem bark chloroform extract has shown promising *in vitro* and *in vivo* trypanocidal activity. The anti-trypanosomal activity observed is attributed to the presence of bioactive compounds in the extract.

ACKNOWLEDGEMENTS

The efforts and technical assistance of Vincent Uper is highly appreciated. The authors wish to express their gratitude to Director, Veterinary Teaching Hospital, Professor E.O. Oyedipe for his invaluable assistance during the course of this study, and Dr. Victor Ahur for analyzing the data statistically.

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BADANIE DZIAŁANIA WYCIĄGU Z KORY *ANNONA MURICATA* PRZECIW ŚPIĄCZCE AFRYKAŃSKIEJ *IN VITRO* I *IN VIVO*

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Streszczenie

Leczenie chemioterapeutykami śpiączki afrykańskiej, tak u ludzi jak i u zwierząt, jest trudne ze względu na powstawanie wielu poważnych objawów niepożądanych oraz wywoływanie zjawiska oporności podczas ich stosowania, co powoduje brak skuteczności i pojawianie się nawrotów tej choroby. Stąd potrzeba znalezienia nowego skutecznego i taniego leku była powodem podjęcia badań przedstawianych w tej pracy. Celem badań było określenie aktywności wyciągu chloroformowego z kory *Annona muricata* stosowanego w warunkach *in vitro* dla trzech stężeń (211, 21,1 i 2,11 mg/ml) przeciwko aktywności *Trypanosoma brucei brucei*. Ponadto w badaniach *in vivo* określano aktywność tego ekstraktu w dwóch dawkach (100 i 200 mg/kg) u szczurów zarażonych pasożytem. Do oceny stanu zwierząt wykorzystano pomiar odpowiednich parametrów hematologicznych po 1 dniu

od ich zainfekowania oraz po 1, 6 i 30 dniu podawania badanego wyciągu. W badaniach *in vitro* we wszystkich zastosowanych stężeniach wyciąg hamował ruchliwość omawianego pasożyta. Ponadto w badaniach *in vivo* ekstrakt całkowicie eliminował obecność *Trypanosoma brucei brucei* u zainfekowanych zwierząt, wykazując w ten sposób obiecującą aktywność przeciwko trypanosomatozie. Zaobserwowane działanie, tak *in vitro* jak i *in vivo*, było być może związane z obecnością związków czynnych charakteryzujących stosowany wyciąg. Analizowany ekstrakt, prawdopodobnie ze względu na obniżenie liczby pasożytów po jego podawaniu, miał także zdolność poprawiania parametrów hematologicznych u zainfekowanych zwierząt. Ze względu na otrzymaną wartość LD_{50} dla zastosowanego wyciągu chloroformowego z kory *Annona muricata*, wynoszącą 1725,05 mg/kg po podaniu dożołądkowym szczurom, można sądzić, że stosowanie badanego wyciągu jest względnie bezpieczne.

Słowa kluczowe: *Annona muricata, śpiączka afrykańska, wyciąg, leczenie, Trypanosoma brucei brucei*