

ANTITRYPANOSOMAL POTENCY OF METHANOL EXTRACT OF *CASSIA AREREH* DELILE ROOT BARK IN ALBINO RATS

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ABSTRACT

The methanol extract of the root bark of *Cassia arereh* Del. was investigated for antitrypanosomal activity *in vitro* and *in vivo* using *Trypanosoma brucei brucei*. *In vitro* antitrypanosomal potency was performed in triplicate in 96 wells microtitre plate, where the infected blood was treated with 1.25, 2.5, 5 and 10mg/ml concentration of plant extract and also with 3.5mg/ml diminazene aceturate and with dextrose saline. The oral lethal dose (LD₅₀) was determined using Lorke's method. Rats were inoculated with *T. brucei brucei* intraperitoneally and treated with the extract orally at 500 and 1000mg/ml (group A and B) while group C and D were given a single dose of 3.5mg/kg of diminazene aceturate and 0.5ml distilled water, respectively. In the *in vitro* study, there was significant extract concentration dependent activity against the parasite motility. The *in vivo* study revealed that treatment with the extract at 500 and 1000mg/ml did not affect the level of parasitaemia as swarming stage were maintained in the groups up to day 11 post treatment, but the diminazene treated group showed no parasitaemia after treatment. The packed cell volume of treated groups also decreased significantly. The oral LD₅₀ was greater than 5000mg/Kg thus the extract has low toxicity and is relatively safe. It was concluded that the methanol extract of *C. arereh* Del. *in vivo* in rats has poor or no antitrypanosomal activity but has significant concentration dependent antitrypanosomal activity *in vitro*.

Keywords: *Trypanosoma brucei*, *Cassia arereh*, Methanol extract.

INTRODUCTION

African Animal Trypanosomosis is a disease complex caused by tse-tse fly transmitted *Trypanosoma congolense*, *Trypanosoma vivax* or *Trypanosoma brucei brucei* or simultaneous infection with one or more of these trypanosomes. African Animal Trypanosomosis is most important in cattle but can cause serious losses in pigs, camels, goats and sheep (Anosa, 1992). The disease (Trypanosomosis) is caused by the protozoa, *Trypanosoma spp*, transmitted through tse-tse fly saliva when they feed on an

animal and can also be transmitted mechanically by tse-tse and other biting flies through the transfer of blood from one animal to another. This protozoon is also the etiologic agent of the disease sleeping sickness in humans. Treatment is associated with many problems such as parasite resistance, drug toxicity and cost. Therefore, there is the need to search and develop new molecules effective against trypanosomes.

Plants have been used thousands of years ago as the major source of drugs both in orthodox and traditional medicine. There are about over 5000 species of plants on earth but only 1 – 10% has been studied for their potential medicinal values (Verpoorte, 2000). Previous studies have reported antitrypanosomal potential of many medicinal plants (Biobaku *et al.*, 2009; Olukunle *et al.*, 2010; Mann *et al.*, 2011). Several well known antiprotozoal drugs are derived from plants and quinine obtained from Cinchona plant and antiminisidine obtained from *Artemisia annua* are both very efficacious antimalarial drugs (Bisignano, 2009).

The plant *Cassia arereh* is a medicinal plant that has been used by traditional medicine practitioner in the management of several ailment including parasitic infections. However, little or no scientific studies have been done on the anti-parasitic activity of this plant. Therefore, this work is carried out to determine the *in vitro* and *in vivo* antitrypanosomal potentials of the methanol extract of *C. arereh* root bark in albino rats.

MATERIALS AND METHODS

Plant Collection and Identification

Fresh leaves, fruits, stem and root-bark were collected from Ngulde district in Borno state, Nigeria and was authenticated by Prof S. S. Sanusi at the Department of Biological Science, University of Maiduguri, Nigeria. A voucher sample (Vet 207A) was preserved at the Veterinary Physiology Laboratory, University of Maiduguri, Nigeria.

Extract Preparation

The sample (root-bark of *C. arereh*) was air dried at room temperature for one week and thereafter was pulverized into fine powder using pestle and mortar. 200g of the extract was mixed with 1 litre of 100% methanol and shaken vigorously. It was then allowed to stand for 24hours and then filtered using Whatman No.1 filter paper. The filtrate was then concentrated to dryness on a rotary evaporator (R201D PEC; Medical USA) maintained at 50°C for 4days.

The methanol extract was then stored at 4°C until needed.

A stock solution of 100mg/ml of the methanol extract of the *Cassia arereh* root-bark was prepared by dissolving 2g of extract in 20ml of distilled water just before use.

Experimental Animal

Adult albino rats weighing between 104.5g and 220g were used for the experiments. They were kept in plastic rat cages and allowed to acclimatize to the laboratory for a minimum period of one week before the commencement of the experiments. They were fed with growers mash (Vital Feeds, Nig. Ltd) and water was provided *ad libitum*. The experiments were conducted in compliance with the international guiding principles for biochemical research involving animals (C.I.O.M.S., 1985).

Acute Oral Toxicity Test

The acute oral toxicity was estimated using Lorke's method (1983) using eleven adult rats of both sexes. The LD₅₀ value was calculated using the formula:

$$LD_{50} = \sqrt{aXb}$$

Where a = least dose that killed a rat while b = highest dose that did not kill any rat

Trypanosome Organism

Albino rats infected with *Trypanosoma brucei* was obtained from the National Institute for Trypanosomosis Research (NITR) Vom, Plateau state. They were maintained on standard laboratory diet and housed in clean cages at room temperature, water was provided *ad libitum*. Blood samples from a donor rat at peak parasitaemia were mixed with dextrose saline and used to perform the *in vitro* and *in vivo* studies.

Inoculation of Experimental Animal with *Trypanosoma Brucei Brucei*

Equal volume of infected blood and 5% dextrose (that is, 1ml of infected blood + 1ml of 5% dextrose) was mixed and sub-inoculated to the experimental albino rats (group A–C, 5 each per group). Each rat received 0.1ml IP using 1ml

syringe. Level of parasitaemia was checked at 4 days interval during the *in vivo* study.

***In Vitro* Test**

From the stock solution (100mg/ml), 1ml was dissolved in 10ml of dextrose saline and then serially diluted to obtain concentrations of 20mg/ml, 10mg/ml, 5mg/ml, and 2.5mg/ml. *In vitro* antitrypanosomal potency was performed in triplicate in 96 wells microtitre plates (Flat bottom). 50 μ l of blood containing about 40 parasites per field was mixed with 50 μ l of the various concentrations of the extract solution to produce extract concentration in the wells of 10mg/ml, 5mg/ml, 2.5mg/ml and 1.25mg/ml. Also sets of negative and positive control in which the infected blood were mixed with dextrose saline and diminazene aceturate respectively were included. The test was carried in Electrical Thermostatic water bath (SM-9043; PEC Medical USA) maintained at 37°C and number of motile parasites was counted at 0, 5, 10 and 15min under light microscope (Leitz Wetzler Germany) at $\times 40$ objective.

***In Vivo* Test**

Twenty adult albino rats (104.5g and 200g) of both sexes were grouped into four groups of five rats each. Those in group A, B and C inoculated IP (intraperitoneally) with *Trypanosoma brucei brucei* and monitored at 4days interval using Herbert and Lumsden method (Herbert and Lumsden, 1976) until a swarming stage were achieved. Those in group A and B were treated with 500 and 1000mg/kg per os (P.O) for 4 days respectively of the methanol extract of *C. arereh* root-bark and those in group C were treated with single dose of 3.5mg/kg BW dose of diminazene aceturate while those in group D administered 0.5ml dextrose saline PO for 4 days. Packed cell volume (PCV) values were determined before injection and then determined together with the level of parasitaemia at day 1, 5 and 9 of treatment with the extract.

Estimation of Parasitaemia

Blood samples obtained from the tail vein of infected rats were examined in wet mount under

light microscope at $\times 40$ magnification. Parasites were determined using the "Rapid matching method (Herbert and Lumsden, 1976). Parasites were counted microscopically per field per unit of blood diluted with dextrose saline. Logarithm values of these counts obtained by matching with the table of Herbert and Lumsden were converted to antilog to get the number of parasite per ml of blood.

Blood Film Preparation

Using quality clean grease-free slide and cover slips, a small drop of well-mixed blood near the right handed end of the glass slide was placed longitudinally on the bench. The cover slip was used to make a clean thin smear with brush border. The slide is dried by waving it in the air and then the slide was labeled. The air dried slide was fixed with methyl alcohol for 3 minutes followed by application of freshly prepared staining solution consisting one part Giemsa's stain to nine parts of neutral distilled water for at least 30minutes, and applied over the whole surface and observed with an oil immersion objective (Baker and Silverton, 1985).

Packed Cell Volume (PCV) Determination

Microhaematocrit centrifuge (Model: SH120-1), fitted with head capable for carrying 24 capillary tubes and a revolution of 12, 000RPM was used for 5minutes. The capillary tube containing anticoagulant was $\frac{3}{4}$ filled with blood samples obtained from the tail vein of the rats. The end of the tube was sealed using plasticine and excess cleared off using cotton wool. The filled tubes were placed in a slot in the centrifuge head with sealed end outward. A special scale, the microhaematocrit reader (Hawksley, England) was used to obtain the Packed Cell Volume percentage and the reading recorded (Baker and Silverton, 1985).

Data Analysis

All data generated during the course of the research were expressed as mean \pm standard deviation (SD) and analyzed statistically by analysis of variance (ANOVA). GraphPad

Instat® (2003) computer statistical software package was used for the analysis and $P \leq 0.05$ was considered significant.

RESULTS

The *In Vitro* Study

Table 1 showed the effect of the various concentrations of the methanol root bark extract of *Cassia arereh* Del. on the *in vitro* motility of *T. brucei brucei*. At the concentration of 1.25mg/ml of the extract, there was no effect on the motility of the parasite one minute post exposure. But after five (5) minutes, the parasites motility decreased significantly ($P < 0.05$) from $7.9 \pm 0.0 \times 10^3$ /ml to $7.4 \pm 0.1 \times 10^3$ /ml which further decreased significantly ($P < 0.05$), to $7.1 \pm 0.1 \times 10^3$ /ml at 10min and by 15min, there was no observed parasite motility. At the concentration of 2.5mg/ml, the parasite motility decreased significantly ($P < 0.05$) to $7.3 \pm 0.1 \times 10^3$ /ml one minute post treatment and at five minutes there were no motile parasite observed. At the concentration of 5 and 10mg/ml, the parasite motility became zero one minute after treatment with the extract, just like that of diminazene treated wells.

The Acute Oral Toxicity Study

The administration of methanol root bark extract of *Cassia arereh* Del. orally to albino rats up to the dose of 5000mg/Kg BW did not record any mortality as shown in Table 2. However, a mild clinical sign of rough hair coat in rats treated with the dosage of 2900 and 5000mg/Kg BW was observed. Thus the LD_{50} was considered to be greater than 5000mg/Kg BW.

The *In Vivo* Study

Table 3 presents the *in vivo* parasite count in albino rats experimentally inoculated with *Trypanosoma brucei brucei* and then treated with the methanol extract of *Cassia arereh* Del. root bark. Parasitaemia was observed on day six post inoculation and swarming stage were observed in all groups on day eight post inoculation. Treatment with the extract at the doses of 500 and 1000 mg/kg BW for four days did not affect the level of parasitaemia as swarming stage were

maintained in the treated groups up to day 20 post inoculation (day 11 post treatment). However the diminazene treated group showed no parasitaemia after treatment.

The Effect on Packed Cell Volume (PCV)

Table 4 showed the effect of the methanol extract of *C. arereh* Del. on Packed Cell Volume (PCV) of infected albino rats with *T. brucei brucei*. At the dosages of 500 and 1000mg/Kg BW of the extract administered, the PCV values decrease significantly ($P < 0.05$) during the course and after withdrawal of treatment up to the end of the experiment (20 days post inoculation). The PCV values of diminazene treated group decrease significantly ($P < 0.05$) from 43.4 ± 1.95 to 37.6 ± 2.79 on day four post treatment but the value raised to 43.4 ± 1.34 by day eleven post treatment (20 days post inoculation). The PCV values of the non infected non treated group increased significantly ($P < 0.05$) from 44.8 ± 0.44 on day zero to 46.4 ± 0.89 on day 20 of the experiment.

DISCUSSION

The *in vitro* study on the plant extract (*Cassia arereh* root bark methanol extract) showed significant antitrypanosomal activity as there was total clearance of the parasite with time. At 1.25mg/ml of the extract, there was no effect on the motility of the parasite one minute post exposure, but after five minutes, the parasites motility decreased significantly ($P < 0.05$) and at the concentration of 2.5mg/ml no motility was observed at five minutes. At the higher concentrations of 5 and 10mg/ml, the parasite motility observed were zero a minute after treatment with the extract showing that the extract indeed has significant antitrypanosomal activity. The extract showed graded concentration dependent activity on the trypanosome motility. This claim may be due to the fact that there was no interference in the system as the plant extract was mixed directly with the blood before examination. And at 5mg/ml and 10mg/ml, their antitrypanosomal

activity was similar to that of diminazene aceturate.

The result of this study showed that the plant extract however, exhibited little or no antitrypanosomal effect *in vivo* as swarm stage of the parasitaemia was maintained in extract treated groups up to the end of the experiment (20 days post inoculation) while on the other hand, diminazene treated group, had total clearance of parasitaemia from the 4th day (day 12 post inoculation) post commencement of treatment without relapse of infection throughout the study. Complete clearance of parasitaemia in rats treated with the control drug diminazene aceturate four days post infection supports previous findings (Ezeokonkwo and Ezeh, 2007). Some of the probable factors that may have led to the failure of the plant extract to exhibit *in vivo* antitrypanosomal activity in the rat could be as a result of the route of administration of the extract, first pass effect, deactivation of the active principle of the extract before reaching blood, metabolic problems, and probably also, specie response (that is, the plant may not be active in rats but may be active in other species of animals).

The acute oral toxicity of the methanol extract of *Cassia arereh* root bark in albino rats up to the dose of 5000mg/Kg BW using Lorke's method (Lorke, 1983) produced no mortality. Thus, the median lethal dose (LD₅₀) of the extract is greater than 5000mg/Kg BW. This indicates that

the extract is relatively safe. It was reported that any substance whose oral LD₅₀ value is above 1000mg/Kg BW is regarded as being of low toxicity or relatively safe (Clarke and Clarke, 1977).

The administration of the extract to the albino rats for four days in this study showed a significant decrease (P<0.05) in the value of the Packed Cell Volume (PCV) at the dosages of 500mg/kg and 1000mg/kg and this continued up to the end of the experiment (20 days post inoculation). Therefore the plant extract could not reverse the decreased PCV in the trypanosome infected condition even though there was significant (P<0.05) increased PCV value of the non infected non treated group. Previous work (Ngulde, 2010) on this plant extract has also resulted in an increase PCV, RBC and haemoglobin count in non infected rats. The plant can therefore be said may contain compounds that stimulate haematopoiesis but this effect was masked because of the high level of decreased PCV values induced by the infection in the treated groups.

In conclusion, the *in vivo* study of the plant extract *Cassia arereh* root bark methanol extract has little or no antitrypanosomal activity, but *in vitro* screening showed significantly higher trypanocidal activity. Further studies on antitrypanosomal activity need to be carried out on other species of animal as *in vivo* effect of extracts may differ among species.

Table 1: The effect of the methanol root bark extract of *Cassia arereh* Del. on the *in vitro* motility of *Trypanosoma brucei brucei*

Extract concentration	Parasite count ($\times 10^3$ /ml) with time					
	0 min	1 min	5 min	10 min	15 min	1 hr
Dextrose saline (5%)	7.9±0.0	7.9±0.0	7.9±0.0	7.9±0.0	7.9±0.0	7.9±0.0
1.25	7.9±0.0	7.9±0.0	7.4±0.1 *	7.1±0.1 *	0.0±0.0 *	0.0±0.0 *
2.5	7.9±0.0	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *
5	7.9±0.0	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *
10	7.9±0.0	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *
Diminazene (3.5mg/ml)	7.9±0.0	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *	0.0±0.0 *

* = (P<0.05) compared to zero minute value respectively for the same concentration range

Table 2: Acute oral toxicity of methanol root bark extract of *Cassia arereh* Del. (Lorke’s method) in albino rats

Phase	No. of rats	Dose (mg/kg)	Clinical signs	Mortality
1	3	10	None	Zero
1	3	100	None	Zero
1	3	1000	None	Zero
2	1	1600	None	Zero
2	1	2900	Rough hair coat	Zero
2	1	5000	Rough hair coat	Zero

Table 3: The effect of the methanol root bark extract of *Cassia arereh* Del. on the *in vivo* parasite count ($\times 10^3/ml$) in albino rats experimentally inoculated with *Trypanosoma brucei brucei*

Days-post inoculation	Days-post treatment	Extract (mg/kg)		Diminazene(mg/kg)	Negative control
		500	1000	3.5	Distilled water
6	-	7.16 \pm 0.5	6.96 \pm 0.6	7.14 \pm 0.5	0
9	0	Swarm	Swarm	Swarm	0
13	4	Swarm	Swarm	0	0
20	11	Swarm	Swarm	0	0

Table 4: The effect of the methanol extract of *Cassia arereh* Del. on Packed Cell Volume (PCV) of infected albino rats with *Trypanosoma brucei brucei*

Treatment Group				
Days-post inoculation	Extract (mg/kg)		Diminazene(mg/kg)	Negative control
	500	1000	3.5	Dextrose saline
0	44.2 \pm 1.30	39.2 \pm 4.15*	41.4 \pm 1.95	45.8 \pm 0.55
9	40.4 \pm 3.58*	35.6 \pm 2.51*	37.6 \pm 2.79*	45.8 \pm 0.55
13	36.8 \pm 3.03*	35.4 \pm 1.34*	39.2 \pm 4.15*	45.6 \pm 0.55
20	34.8 \pm 2.59*	35.4 \pm 1.34*	39.2 \pm 4.15*	46.4 \pm 0.89 ^a

* = significant (p<0.05) decrease compared to respective day zero

^a = significant (p<0.05) increase compared to day zero

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