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In vitro and bioinfectivity evaluation of *Trypanosoma brucei brucei* challenged with raw and differently processed *Moringa oleifera* seeds

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Target Audience: *Animal Scientist, Veterinarians and Livestock farmers.*

Abstract

Raw Moringa oleifera (M.) seeds possess mild antitrypanosomal properties and processing could improve its potency. The in vitro and bio-infectivity of Trypanosoma brucei brucei in raw, boiled and fermented M. seeds was evaluated in a completely randomized design. Motility rates of trypanosomes were observed at contact and intervals of fifteen minutes up to one hour of incubation at the in vitro trial. Tube 1 (control) contained 0.3mL parasitized blood and 0.7mL Ringer's solution. The 2nd, 3rd and 4th tubes contained 0.3mL parasitized blood + 0.4mL Ringer's solution + 0.3mL each of 50 mg/mL of raw, boiled and fermented M. seeds respectively. At the bioinfectivity trial, twelve mice approximately 30 days old were randomized into four groups and infected with trypanosomes collected from the tubes in triplicates. Descriptive statistics, ANOVA, DMRT, and line graphs were used for data analysis. Results revealed that at 45th minute of incubation, motility rates of parasites in tubes with raw and fermented M. seeds were ($p<0.01$) lower than boiled M. seeds and negative control. At 60th minute, all tubes containing M. seeds had zero motility of parasites except control. Parasitaemia graph showed flat line all through 30 days observation for mice in group 3 while groups 2 and 4 had fluctuating parasitaemia trend. Group 1 had upward trend till peak parasitaemia. Weights and PCV of mice across groups were insignificant ($p>0.05$). Pre patency was insignificant across groups while group 3 had ($p<0.05$) higher survival time. Boiled M. seeds had best inhibitory potentials of trypanosomes.

Key words*: Parasitaemia; Process; Inhibition; Moringa; seeds*

Description of Problem

Trypanosomiasis is a disease caused by flagellated protozoan blood parasites belonging to the genus *Trypanosoma* (1). The disease is prevalent across several geographical regions such as Africa, Asia, Latin America, Central America and the Carribean (2, 3, and 4). In Africa, common pathogenic *Trypanosoma species* of economic importance are *Trypanosoma brucei brucei (T.b.brucei), Trypanosoma congolense (T.congo), Trypanosoma vivax (T.vivax), Trypanosoma evansi (T.evansi), Trypanosoma simiae (T.simiae), Trypanosoma theileri (T. theileri)* and *Trypanosoma equiperdum (T.equiperdum)*

(5). *T. b.brucei* belongs to the trypanozoon subgenera and is mainly transmitted by the vector *Glossina species* (Tsetse flies) (6). The parasite is morphologically distinguished by the position of the relatively smaller kinetoplast which is at the base of the flagellum and a pointed posterior end (7). Its blood form has a slender, elongated and streamlined shape resembling a tear drop.

The trypanosome causes infections of economic significance to animals belonging to the following families; Bovidae, Girafidae, Hippopotamidae, Pantherinae, Rhinocerotidae and Canidae (5). Its pathogenic effects ranges from reduced parturition rate, abortion, loss of condition, high mortality rate, low meat and milk yield (8). There are limited veterinary trypanocidal drugs (Diminazene aceturate, Isometamidium chloride and Homidium salts) with several reports on their lapses such as toxicity, relapse due to resistant trypanosome strains and high cost making it unaffordable for poor rural livestock farmers. These have necessitated the screening of several herbal plants. Researchers over the years have been experimenting on these plants for their antitrypanosomal claims by rural local healers that use plant parts in unconventional methods of treatment of sick animals (9, 10, 11, 12, 13, 14, 15) to find effective alternative treatments for Trypanosomiasis. Among these plants, the frequently screened plant is *Moringa oleifera (M. oleifera)*. The use of *M. oleifera* plant parts such as leaves, stem bark and seeds have been reported to have some level of inhibitory antitrypanosomal properties (16, 17, 18, 15) when used as a herbal treatment for Trypanosomiasis. However, none of these research findings can stand as a replacement for available trypanocidal drugs in the market.

There are reports of raw *M. oleifera* containing antinutrients that interfere with digestion and utilization (13) but heat processing of seeds has been reported to improve nutrient utilization of the seeds by animals (19). Processing methods such as boiling and fermentation of *M. oleifera* seeds may also proffer better antitrypanosomal potential in the treatment of Trypanosomiasis. Information on trypanocidal potentials of these differently processed *M. oleifera* seeds are scarce and this study is aimed at discovering the most effective treatment among raw, boiled and fermented seed meal in *in vitro* and its bioinfectivity trials in mice experimentally infected with the *T. brucei brucei* challenged with the differently processed *M. oleifera* seeds after one hour of incubation at room temperature.

Materials and Methods

The Study Area

The study was carried out at a laboratory facility in Livestock Division of Nigerian Institute for Trypanosomiasis Research (NITR) Vom, with geographical location between latitude 9°72''N and longitude 8°79'E on an altitude of 4200 feet (1280 m) above sea level in the Guinea savannah zone of West Africa. The relative humidity ranges from 78 % during the raining season and 22 % within the dry season, with mean daily environmental temperature ranges of 17 °C-28.6 °C (20, 15).

Collection and Processing of Test Ingredient

Mature and dry *Moringa oleifera* pods containing seeds were harvested within Makurdi Local Government Area of Benue State, Nigeria, with geographical location between latitude 7°75''N and longitude 8°56'E (15). A total of 300 g of *Moringa oleifera* seeds was removed from their pods and divided into 3 equal portions where the 1st portion in its raw form was air-dried in the laboratory for 3 days after washing with

water. The 2nd portion was soaked in 1L of water and allowed to ferment in an enclosed flask for 24 hours (21); which was then drained, air-dried at room temperature for 3 days. The 3rd portion was boiled using pressure pot with 1 L of water for 10 minutes of whistling; which was then drained after cooling, air-dried at room temperature for 3 days. All the seed portions were pulverized separately using an electric blender. The raw and differently processed pulverized *Moringa* seeds were then stored separately in labeled polythene bags until required.

Experimental Design and Procedure

***In vitro* antitrypanosomal phase**

Stabilate of the trypanosomes were obtained from the parasitology division of NITR Vom and inoculated into laboratory rodents as donor. 0.4 mL of Ringer's solution and 0.3 mL of the *T. brucei brucei* diluted blood (containing approximately 6.32×10^6 trypanosomes per mL of blood) was mixed and dispensed (0.23 mL) in triplicates into 4 conical tubes ($A_{1,2,3}$, $B_{1,2,3}$, $C_{1.2.3}$ and $D_{1,2,3}$) containing Ringer's solution and motile *T. brucei brucei* diluted blood with normal saline. 50 mg each of raw, boiled and fermented *Moringa oleifera* seed meal (MSM) was collected using a scoopula and dispensed in separate labeled conical flasks and reconstituted with 1 mL distilled water. The solution was then filtered through a Whatman filter paper (number 42) into a 50 mL conical flask. 0.1 mL each of the filtrate was aspirated from each conical flask using a 1 mL syringe and dispensed into tubes 2 to 4 in triplicates for the raw, boiled and fermented 50 mg/mL concentrations of MSM respectively. At contact, drops of the contents from each tube was aspirated with a micro pipette and dispensed on a clean grease free slide, covered with a cover slip (wet film method) and viewed under a × 40 objective for *T. brucei brucei* motility or cessation of motility, which was then incubated at room temperature and monitored at intervals of 15 minutes using a stop watch until the 60th minute when the *in vitro* trial was terminated. The cessation or increase in motility was rated as; 0- no motility, 1- sluggish, 2- active. Cessation in motility signified antitrypanosomal action.

Bioinfectivity phase

For Bioinfectivity test, 0.1 mL contents of tubes A, B, C and D replicated thrice was inoculated intraperitoneally into 12 healthy, weaned female albino mice respectively that were approximately 30 days old and arranged into 4 groups with 3 albino mice each using the Completely Randomized Design (CRD). The Bioinfectivity parameters observed for inhibition effects were daily parasitaemia using drop of blood collected from the tail vein using the wet film method described by (22). Weekly % Packed Cell Volume (PCV) was determined by the microhaematocrit method were 0.1 mL of blood was milked from snip cut of the tip tail vein. Live weights (g) was collected by placing a mouse restraining box on a digital scale and tare, before the weight of each mouse was taken separately on a weekly basis. Prepatency and survival time (days) of each mouse per group was recorded on a parasitaemia chart. This was carried out for duration of 30 days.

Ethical clearance

Approval on the use of laboratory animals to be infected with *T. brucei brucei* was obtained from the Animal Care and Use Ethical Committee of the College of Veterinary Medicine, Joseph Sarwuan Tarka University, Makurdi with reference number: **JOSTUM/CVM/ETHICS/2023/8** before commencement of the study.

Experimental Animals and Management

A total of 12 healthy female weaned swiss albino mice weighing 20-30 g were bred in a secured laboratory at the Livestock Division

NITR, Vom for the bioinfectivity trial from parent mice bought from the small laboratory animal colony of the Parasitology Division NITR, Vom. Each bred female mouse was kept separately in 12 aluminum fabricated mouse cages fitted with a feeder and drinker. Dry wood shavings used as bedding were changed weekly. Each mouse was marked with picric acid for ease of identification. The mice were fed with broiler super starter (Chikun®) poultry feed and given potable drinking water *ad libitum*.

Data analysis

Data generated from the experiment were subjected to descriptive statistics and analyzed with One-way Analysis of Variance (ANOVA) at $p<0.05$ and $p<0.01$ using the Statistical Package for Social Sciences (SPSS Version 24). Duncan's Multiple Range Test (DMRT) was used to compare the significant treatment means using same SPSS.

Results

The results of the *in vitro* trial revealed that at 45th minute of incubation, motility rates of parasites in tubes with raw and fermented *Moringa* seeds were significantly ($p<0.01$) lower than boiled Moringa seeds and negative control. At 60th minute, all tubes containing *Moringa* seeds had zero motility of parasites except negative control (Table 1).

Parasitaemia graph showed flat line all through 30 days observation for mice in group 3 (challenged with *T. b. brucei* tube containing the boiled aqueous *Moringa* seed extract) while mice in groups 2 and 4 challenged with *T. b. brucei* tubes containing raw and fermented aqueous *Moringa* seeds had fluctuating parasitaemia trend. Negative control (group 1) had upward trend till peak parasitaemia (Figure 1). Group 3 had significantly ($p<0.05$) higher survival time while pre patency was insignificant ($p>0.05$) across all the groups (Table 2).

Live weights and packed cell volume of mice across groups were insignificant ($p>0.05$) as shown in Table 2. Dry film stained parasites as revealed under the × 40 objective showed massive, nil, nil and scanty *T. brucei brucei* per slide for groups 1, 2, 3 and 4 respectively (Plates 1, 2. 3 and 4).

Table 1. *In vitro* motility rates of *Trypanosoma brucei brucei* reaction with negative control, raw, boiled and fermented *Moringa oleifera* seeds

Incubation Time (minutes)	Negative Control T1	Differently processed pulverized seeds (50 mg/ml) in distilled H_2O			p-value	SEM
		T2	T3	T4		
At Contact	2.00^{b}	2.00^{b}	2.00^{b}	1.33^{a}	0.02^{*}	0.24
15	2.00	1.70	2.00	1.30	0.08	0.33
30	1.70	1.70	2.00	1.00	0.09	0.53
45	2.00^{b}	0.00^{a}	1.70^{b}	0.30^{a}	0.00^{**}	0.33
60	2.00	0.00	0.00	0.00	-	-

a and b means in the same row with different superscripts are significant at $p<0.05^{*}$ and highly significant at $p<0.01^{**}$, 0.00 no motility, 1.00 sluggish, 2.00 active mean motility rates. T1 (control group) = 0.4 mL Ringer's solution + 0.3 mL Parasitized blood, T2 (group 2) = 0.4 mL Ringer's solution + 0.3mL Parasitized blood + 0.3 mL of 50 mg/mL concetration extract from raw *M. oleifera* seeds, T3 (group 3) = 0.4 mL Ringer's solution + 0.3 mL Parasitized blood + 0.3 mL of 50 mg/mL concentration extract from boiled *M. oleifera* seeds, T4 (group 4) = 0.4 mL Ringer's solution + 0.3 mL Parasitized blood + 0.3 mL of 50 mg/mL concentration extract from fermented *M. oleifera* seeds.

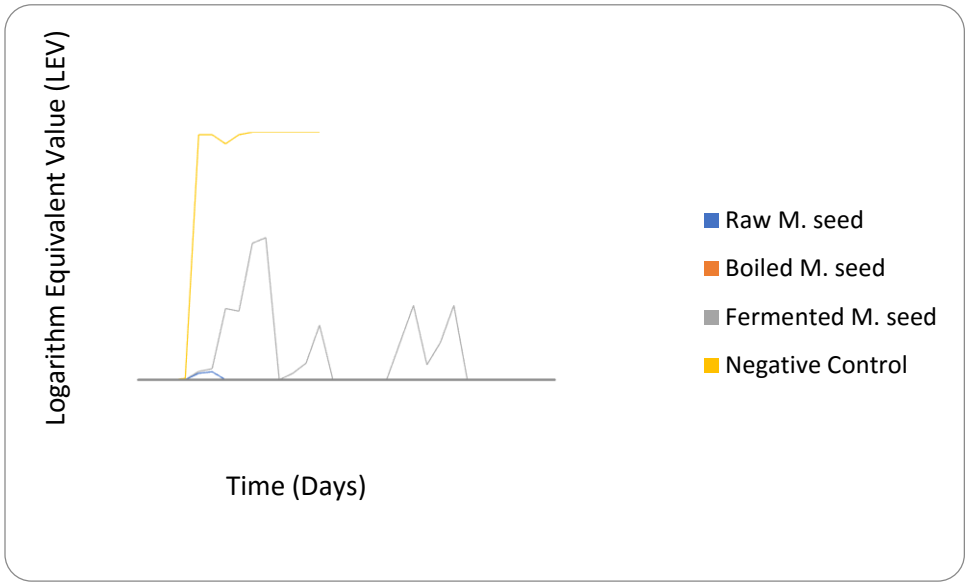
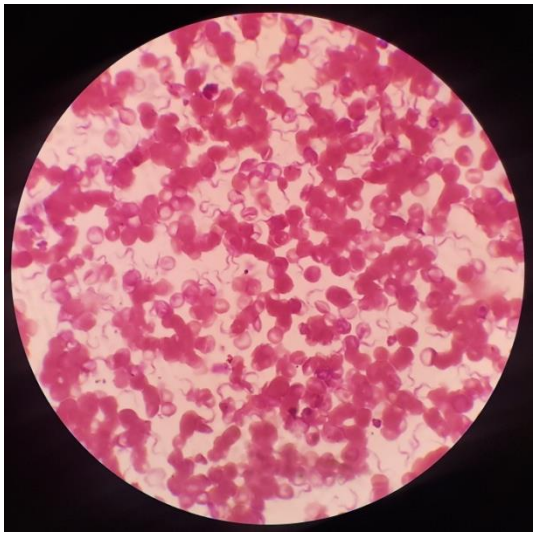


Figure1. Logarithm Equivalent Value (LEV) of *Trypanosoma brucei brucei* challenged with 50 mg/mL each of raw (group 2), boiled (group3), fermented (group 4) *Moringa oleifera* seed and the Negative Control (group 1) within 60 minutes of incubation and infected in albino mice with parasitaemia observed for 30 days using a × 40 objective

Table 2: Growth Performance, packed cell volume (PCV), and pre-patent period of albino mice infected with *Trypanosoma brucei brucei* challenged with 50 mg/mL concentration of raw, boiled and fermented *Moringa oleifera* seed

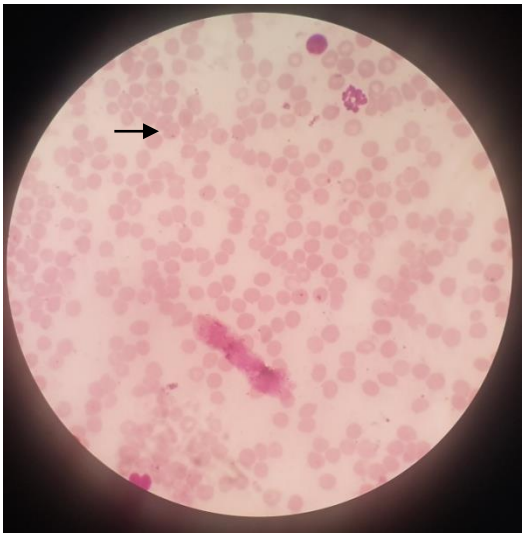
Parameters	Groups					
	1	**2**	**3**	**4**	**p-value**	**SEM**
Initial weight (g)	22.67	24.80	22.07	26.07	0.58	1.07
Final weight (g)	21.80^{b}	28.37^{a}	26.40^{ab}	24.03^{ab}	0.03^{*}	1.07
Weight gain/loss (g)	-0.43	3.57	4.33	-2.04	0.33	1.40
Survival time (days)	10^{b}	21^{ab}	30^{a}	25^{ab}	0.03^{*}	3.18
*Number in group	3	3	3	3	-	-
*Number dead (%Mortality)	3(100)	1(33.33)	0(0)	1(33.33)	-	-
PCV (%)	50.67	55.20	51.13	48.36	0.67	1.81
Pre-patent period (days)	6	2	0	5	0.35	0.94

a and b means in the same row with different superscripts are significant at $p<0.05^{*}$ and highly significant at $p<0.01^{**}$, *****not subjected to statistical analysis, group 1 mice infected with *Trypanosoma brucei brucei* after 60 minutes (negative control), group 2 mice infected with *Trypanosoma brucei brucei* after 60 minutes of contact with raw *Moringa* seed concentrate (50 mg/mL), group 3 mice infected with *Trypanosoma brucei brucei* after 60 minutes of contact with boiled *Moringa* seed concentrate (50 mg/mL), group 4 mice infected with *Trypanosoma brucei brucei* after 60 minutes of contact with fermented *Moringa* seed concentrate (50 mg/mL), SEM Standard Error of Mean.



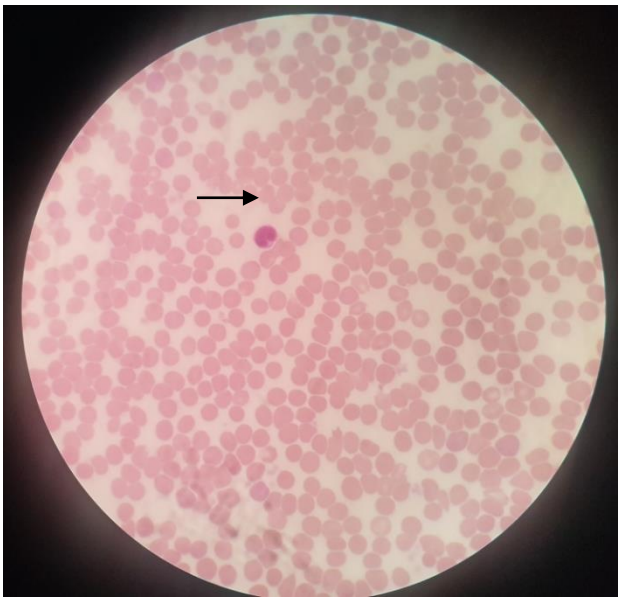
Key: → Red blood cells⟹ *Trypanosoma brucei brucei*

Plate1: Photomicrograph of Negative Control (group 1) *Trypanosoma brucei brucei* inoculated in mouse (Giemsa stain ×100 objective using oil immersion).



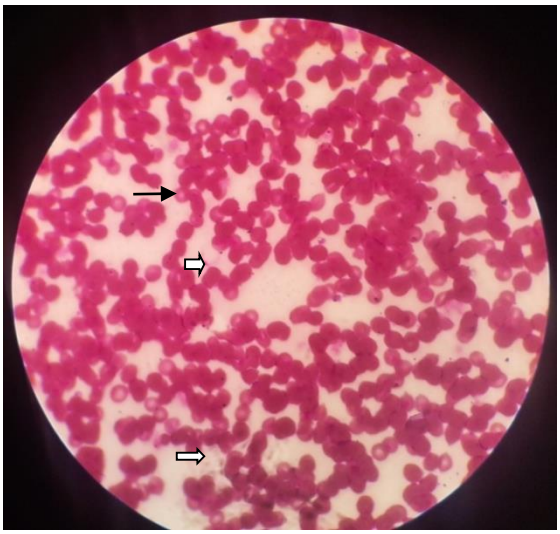
Key: → Red blood cells.

Plate 2: Photomicrograph of raw (group 2) *Moringa oleifera* seed aqueous extract challenged *Trypanosoma brucei brucei* inoculated in mouse. (Giemsa stain ×100 objective using oil immersion).



Key: → Red blood cells.

Plate 3: Photomicrograph of boiled (group 3) *Moringa oleifera* seed aqueous extract challenged *Trypanosoma brucei brucei* inoculated in mouse. (Giemsa stain ×100 objective using oil immersion).



Key: → Red blood cells, ⇨ *Trypanosoma brucei brucei*

Plate 4: Photomicrograph of fermented (group 4) *Moringa oleifera* seed aqueous extract challenged *Trypanosoma brucei brucei* inoculated in mouse. (Giemsa stain ×100 objective using oil immersion).

Discussion

The *in vitro* and bioinfectivity trial in this study were not consistent. It showed that direct contact at forty fifth minutes of incubation with the raw and fermented aqueous *M. oleifera* extracts had highly ($p<0.01$) significant immobilization effect on *T. b. brucei* , while it took 60 minutes of incubation for the boiled aqueous *M. oleifera* seed extract to completely immobilize the trypanosomes. On the contrary, infectivity occurred for the control, raw and fermented aqueous *M. oleifera* seed extracts while only the boiled aqueous *M. oleifera* seed extract revealed complete inhibition of infection to the mice. This finding disagrees with the report of (10) that stated consistency in activities observed *in vitro* and *in vivo* for methanol and aqueous extracts of *M. oleifera*'s various plant parts. Amuga *et al*. (16) Reported mild *in vivo* antitrypanosomal properties of raw aqueous *M. oleifera* seed extract in rats infected with *T. b. brucei* which concurs with the infectivity status of raw aqueous *M. oleifera* seed extract in mice infected with *T. b. brucei*.

These variations in motility observations of *T. b. brucei* in *in vitro* and *in vivo* conditions may have occurred due to the contrasting environmental differences of both mediums where pH, temperature, osmotic pressure, nutrients and phyto-nutrients availability varied. The boiled aqueous *M. oleifera* seed extract at 60 minutes of incubation with *T. b. brucei* which immobilized the trypanosomes and completely impeded infection of the mice in group 3 suggests that the boiled aqueous seed extract may have contained phyto-compounds activated by boiling under pressure to be responsible for this complete immobilization of the *T. b. brucei* that were absent or inactivated in the raw and fermented aqueous seed extracts that supported infectivity. According to the report of (23) methanol extract of raw *M. oleifera* seed extract at 5 mg/mL concentration immobilized *T. b. brucei* at 5 minutes of incubation. This finding corroborates with the *in vitro* observation in the current study of aqueous extract of raw and fermented *M. oleifera* seed extract at 50 mg/ml concentration immobilizing *T. b. brucei* at 45 minutes of incubation.

The similarity in PCV across groups observed in this experiment for mice infected with *T. brucei brucei* disagrees with the report of (23) who observed a significant ($p<0.05$) decrease in PCV of rats *in vivo*. This observation is unusual as anaemia is known to be a cardinal sign for Trypanosomiasis. Rapid replacement of destroyed red blood cells in the mice circulatory system may have been the reason for normal PCV values observed in this study. The insignificant weight loss and prepatency of mice infected with *T. b. brucei* agrees with the findings of (23) in rats infected with *T. b. brucei*. The survival of all mice in group 3 and in healthy state has proven the superiority of boiled aqueous *M. oleifera* seed extract as a potent antitrypanosomal candidate over raw and fermented aqueous *M. oleifera* seed extract.

Conclusions and Application

1. The highly significant antitrypanosomal potency of raw and fermented *Moringa oleifera* seed at the forty fifth minute of incubation varied completely from the bioinfectivity outcome.
2. Boiled *Moringa oleifera* seed conferred better antitrypanosomal properties in mice when compared with raw and fermented seeds by completely inhibiting the trypanosome infection.
3. Further investigation on graded increase dose levels of boiled *Moringa oleifera* seed as feed additive can be carried out in higher

laboratory animals susceptible to *T. b. brucei* infection.

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