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Original Article

Trypanocidal Potentials of *Moringa oleifera* and *Azadirachta indica* Leaves in *In-vitro* Condition against *Trypanosoma brucei brucei*

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ABSTRACT

The antitrypanosomal activity of ethyle acetate leaf extract of *Moringa oelifera* and *Azadirachta indica* on *Trypanosoma brucei brucei* was evaluated in *in vitro* conditions. Three concentrations (0.3 mg/ml, 0.5 mg/ml and 1.0 mg/ml) of each extract were tested against a negative control. The activity was recorded at 15 min., 30 min., 45 min. and 60 min. of incubation with extracts at 37°C. The trypanosomes showing undulating movements were taken as live. The result indicated that both extracts showed statistically significant (p< 0.05) reduction in the trypanosomes counts, compared to control. Among the tested extracts *Azadirachta indica* was more potent against *Trypanosoma brucei brucei* than the *Moringa oleifera* extract. In both cases the effect was time and dose dependent.

Keywords: *Trypanosoma brucei brucei*, *Azadirachta indica, Moringa oleifera,* African animal trypanosomiasis, Tsetse fly etc.

1. INTRODUCTION

Trypanosomiasis is a debilitating parasitic disease of both human and animals [1]. This disease is caused by several species of protozoan parasites of the genus *Trypanosoma*. The disease can affect various species of mammals, butmore prevalent in cattle. It is mainly caused by *Trypanosomabruceirhodensiensis*, *Trypanosomabruceigambiensis,* and *Trypanosoma brucei brucei* [2].

The disease is transmitted by a vector known as Tsetse fly (*Glossinaspp*). Tsetse fly transmitted trypanosomiasis is

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classically acute or chronic disease that causes intermittent fever accompanied by anemia, edema, lacrimation, enlarged lymph nodes, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive and/or nervous signs with emaciation and eventually death in chronic forms [3].

The epidemiology of vector-borne diseases are complex due to variability in the ecology of the parasites, vectors and hosts. Tsetse-borne trypanosomiasis is a widespread protozoan disease affecting wildlife, livestock and people in sub-Saharan Africa, with a range of pathologies, from chronic and long lasting to acute and rapidly fatal, depending on circumstances [4]. The epidemiology of African Animal Trypanosomiasis (AAT) in Tsetse infected areas of Africa is determined by four biological factors, namely: trypanosomes, tsetse flies, reservoir hosts and livestock. However, cattle are the domestic species in which the disease is most frequently diagnosed and treated. When dealing with the tsetse-transmitted trypanosomiasis, much depends on the distribution and the vectorial capacity of *Glossina* species responsible for transmission. Among the three groups of *Glossina*, the savannah and riverine are the most important, since they inhabit areas suitable for grazing and watering. Biting flies may act as mechanical vectors, but their significance in Africa is still undefined [5].

African animal trypanosomiasis (AAT) is a very important disease of domestic livestock in sub-Saharan Africa. According to the Food and Agriculture Organization of the United Nations (FAO), it is probably the only disease which has profoundly affected the settlement and economic development of the major part of a continent. Trypanosomiasis affects the health and productivity of livestock. It occurred in 37 sub-Saharan countries covering about 9 million km^2, an area that corresponds approximately to one-third of the Africa's total land area [6]. An estimated 45 to 60 million cattle and small ruminants are at risk of trypanosomiasis infection [7-8]. FAO estimates that about three million cattle die each year due to AAT [9]. Other valuable livestock, such as camels, also suffer from trypanosomiasis [10].

Thirty-five millions doses of trypanocides are being administered each year to protect livestock in tsetse infected areas [11]. Direct losses due to trypanosomiasis are estimated to amount between US$ 1-1.2 billion each year whereas the indirect impact of AAT on agriculture in sub-Saharan Africa exceeds this amount. Direct costs due to AAT involve decreased livestock productivity (mortality, fertility, milk yield, ability to work as traction animals) to which can be added expenditure on controlling the disease [12]. A pondered evaluation extrapolated for the total tsetse-infested lands values the total losses, in terms of agricultural Gross Domestic Product, at US$ 4.75 billion per year [9].

The Chemotherapy of African Trypanosomiasis is centered on few drugs (Steverding, 2008). The available drugs are only effective against some species, and less effective againstothers.

At present, the management of this disease is mainly through chemotherapy. The current drugs used as veterinary trypanocides include Diaminazeneaceturate (Berenil) and also methamidium (Samorin). Resistance to both therapeutic agents has however been documented in field studies; therefore the present and urgent need to develop efficacious chemotherapeutic agents from locally available ethno-medical plants for trypanocidal use [13-14]; thus this study was aimed to study trypanocidal efficacy of *Moringa oliefera* and *Azadirachta indica*, leaves extract in *in-vitro* condition.

2. MATERIALS AND METHODS

Sample collection

Albino Mice

Four albino mice were obtained from the animal house of the Nigeria Institute for Trypanosomiasis and Onchocerciasis, Kaduna state, Nigeria. The animals were of both sexes. All the animals were kept in a ventilated place and they were fed with grower marsh (Fitzer Lagos, Nigeria) and water. The mice when required, were used as donors of trypanosomes for the *Invitro* analysis.

Trypanosome Stock

The test organism *Trypanosoma brucei brucei* (Federe strain) was obtained as cryostabilates from the vector and parasitological studies department, Nigerian Institute for Trypanosomiasis Research; Kaduna. After thawing, a blood suspension of the stabilates in phosphate buffer saline was inoculated into mouse. The organisms (parasites) were maintained by serial passages in mice.

Plant Collection and Identification

Fresh leaves of *Moringa oleifera and Azadirachta indica* were collected from garden in Usmanu Danfodiyo University, Sokoto and was properly identified. The leaves were dried under 95% canopy/shade (at room temperature) with a circulating air flow, this is done to enable the plant retain all the chemical compounds present and then pounded into powder form using a laboratory mortar.

Extraction of *Moringa oleifera* and *Azadirachta indica*

One hundred grams (100)g of each of the leaves powder were weighed and transferred into 500ml glass beaker and 250ml of ethyl-acetate was added to each. It was allowed to extract by maceration for 72hrs. After that, it was filtered with cloth, and then with a double filter paper. The filtrate was collected in clean crucibles and covered with net and allowed it to evaporate at room temperature. The concentrates were transferred into a cleaned weighed sample bottle. The extract was stored at room temperature [15].

Experimental Procedure

Determination of parasitamia

The trypanosome count in the infected rats was determined by the rapid matching method of [16]. Blood collected by tail snip or cardiac puncture was placed on a clean grease free glass slide and a cover glass placed over it; the blood spread

into thin circular film. The slide was placed in the light microscope and examined at x400 magnification. The distribution of the trypanosomes among the red blood cells (RBCs) was matched against the lumsden's chart and approximate number of trypanosomes per milliliter of blood was estimated [16].

Parasite Harvest Using Cardiac Puncture

The rat with rising parasitaemia of 10-40 parasites per microscopic field was dissected after Chloroform anesthesia, the animal was placed on a dissecting board, the hind and the fore limbs were pinned. Dissecting scissors was used to dissect the animal;EDTA 0.2ml solution in a 2ml syringe was spread on the heart and internal surface to avoid coagulation of the blood. The blood was collected through cardiac puncture into the syringe containing EDTA solution, then transferred into clean EDTA container and gently mixed together to prevent clotting of the blood [17].

Media of the trypanocidal activity

Phosphate buffered saline (pH 7.4) supplemented with 1% glucose (w/v) (PBS-G) was used as a media for the anti-trypanosomal assay. PBS-G has been reported to support trypanosome survival for about 4hrs in *in-vitro* conditions. The media constituted was used to dilute the parasitized blood and also as solvent for the dissolution of the extracts and drugs [18].

Reconstitution of solutions of the plant extracts and reference drug

Solutions of the plant extracts and drug were reconstituted in PBS-G. Stock solutions of the respective plant extracts were prepared by dissolving 5mg of the extract in 5ml, 10ml and 15ml of PBS-G; subsequently, the stock solution was serially diluted in PBS-G to yield extracts with concentrations of 0.3mg/ml, 0.5mg/ml and 1.0mg/ml respectively. The various concentrations of Diminazeneaceturate was reconstituted, concentrations ranging from 1 mg/ml to 0.03125 mg/ml [19].

***In vitro* anti-trypanosomal assay**

Fifty (50) µl of the reconstituted solutions of the extract, as well as the reference drug, were separately dispensed in triplicate into wells of a 96-well microtitre plate. To each of these wells was added, 50µl of the blood suspension containing *T. bucei brucei* and gently mixed together. Control wells containing only 50µl PBS-G and 50µl blood suspension were also included. Wet smears were prepared from each of these wells at 15minutes, 30minutes, 45minutes and 60 minutes post-incubation; each smear was examined in the light microscope (X400 magnification) for trypanosome activity and motility was counted over three fields of view per smear, with a total of nine observations per concentration. No movement in the trypanosome body or no undulating movement was taken as evidence of death. Each observation was compared against that from the control wells [17].

Data was analyzed with Statistical Package for social Science (SPSS) version 20 software and graphs were plotted using Microsoft excel. Descriptive data were presented as mean ± standard deviation of trypanosome counts. The mean trypanosome count per concentration of extract at the various time intervals of incubation were compared using one way analysis of variance (ANOVA) at significance level of $p = 0.05$. The t-test was used to compare the effect of each concentration on the two solvent extracts for mean trypanosome count.

3. RESULTS

The ethyl acetate leaf extracts of *Moringa oleifera* and *Azadirachta indica*, on African trypanosomes showed very effective in *In vitro* condition. Three concentrations of the extracts were used; 0.3 mg/ml, 0.5 mg/ml and 1.0 mg/ml respectively. Observations were taken at four different time intervals: 15, 30, 45, and 60 minutes post incubation, respectively. Reduction in trypanosome count was recorded as an index of antitrypanosomal activity. Among the used plants, *Moringa oleifera* was more effective at lower exposure period (15 min., 30 min. and 45 min.) however, *Azadirachta indica* was more effective at 60min. exposure period. In both cases, the trypanocidal effect of extracts were time and dose dependent.

Effect of Ethyl Acetate Leaf Extract of*Moringaoleifera* on Trypanosome Count:

The effect of the different concentrations of ethyl acetate leaf extract of*Moringaoleifera* on the mean trypanosome count in wells of the microtitre plate was presented (Table 1). At each time interval, there was a significant difference ($p< 0.05$) in trypanosome count between microtitre wells with the extract compared to the control wells (Table 1). Furthermore, the result indicated that for the respective time interval of observation, the effect of the extract followed a concentration dependent pattern, with higher concentrations of the extract exhibiting greater effect on trypanosome count (Fig. 1). At 45 and 60 minutes post incubation, the results showed that there were no statistically significant differences (1.0 mg/ml and 0.5 mg/ml) on trypanosome count.

Table 1: Effect of the Different Concentrations of Ethyl Acetate Leaf Extract of *Moringa olifera* on the Mean Trypanosome Count

Concentrations (mg/ml)	Mean trypanosome count per field			
	15 min	30 min	45 min	60 min
1.0	7.33±2.1[a]	5.67±1.5[a]	5.67±0.6[a]	3.67±0.6[a]
0.5	22.7±3.1[b]	14.7±2.5[b]	8.67±0.6[a]	4.67±1.5[a]
0.3	29.7±2.5[c]	20.3±3.2[c]	13.7±2.1[b]	8.67±2.5[b]
0.0 (control)	31.3±3.1[c]	28.3±2.1[d]	26.3±3.1[c]	22.3±1.5[c]
***F* (3, 8)**	48.981	46.833	69.605	78.471
***p* value**	< 0.001*	< 0.001*	< 0.001*	< 0.001*

Values are given as mean ± standard deviation. In each column, values with different superscripts have statistically significant different ($p< 0.05$)

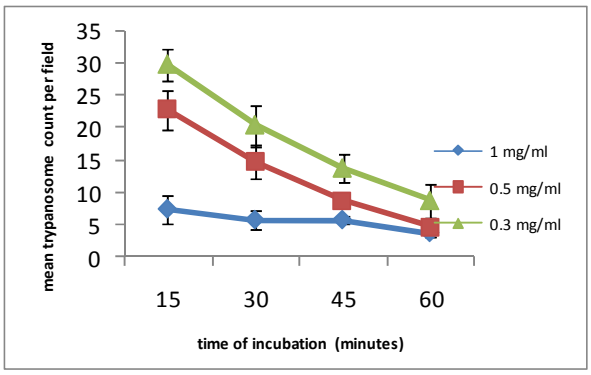

Fig 1: Effect of three concentrations of ethyl acetate leaf extract of *Moringa olifera* on *Trypanosoma brucei brucei* at four intervals of incubation. The effects of the extract increased with time of incubation.

Effect of Ethyl Acetate Leaf Extract of *Azadirachta indica* on Trypanosome Count:

There were statistically significant differences ($p< 0.05$) between the mean trypanosome count in wells of the microtitre plate containing the *Azadirachta indica* extract compared to the control well over the all four time intervals of incubation (Table 2). The effect of the extract on trypanosome was concentration and time dependent (Fig 2). At 60 minutes post incubation, the results showed that 1.0 mg/ml and 0.5 mg/ml of the extract exhibited significant effect on trypanosome count.

Table 2: Effect of the Different Concentrations of Ethyl Acetate Leaf Extract of *Azadirachta indica* on the Mean Trypanosome Count

Concentration (mg/ml)	Mean trypanosome count per field			
	15 min	30 min	45 min	60 min
1.0	14.7±4.2^{a}	11.0±3.6^{a}	7.33±2.1^{a}	0.33±0.6^{a}
0.5	28.0±2.6^{b}	21.3±1.5^{b}	13.3±3.1^{b}	0.33±0.6^{a}
0.3	30.7±2.3^{b}	27.7±2.5^{c}	25.7±2.9^{c}	14.3±2.1^{b}
0.0 (control)	31.3±3.1^{b}	28.3±2.1^{c}	26.3±3.1^{c}	22.3±1.5^{c}
***F* (3, 8)**	18.724	29.791	33.66	194.182
***p* value**	0.001*	< 0.001*	< 0.001*	< 0.001*

Values are given as mean ± standard deviation. In each column, values with different superscripts have statistically significant different ($p< 0.05$)

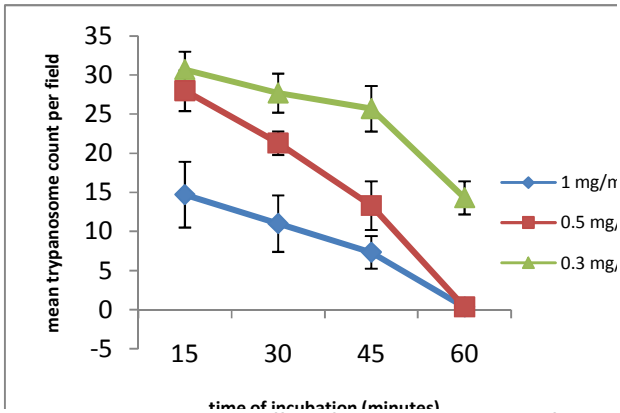

Fig 2: A chart showing the effect of three concentrations of ethyl acetate leaf extract of *Azadirachta indica* on *Trypanosoma brucei brucei* at four intervals of incubation. The effects of the extract increased with time of incubation.

Comparison of the Effect of Ethyl Acetate Leaf Extract of *Moringa olifera* and *Azadirachta indica* on Trypanosome Survival

Table 3 and Fig.3 showed the comparative effects of both *Moringa oleifera* and *Azadiractha indica* on trypanosome survival. It was observed that at equal concentrations of both extracts, *Moringa oleifera* demonstrated significant trypanocidal effects compared to *Azadiractha indica*, especially between 15 and 45 minutes of incubation. That could be seen as fewer number of surviving trypanosomes in wells of the titre plate containing the *Moringa oleifera* extract.

Table 3: Comparative Effect of Ethyl Acetate Leaf Extracts of *Moringa olifera* and *Azadirachta indica* on *Trypanosoma brucei brucei*

Concentration (mg/ml)	Mean trypanosome count per field							
	Moringa olifera				*Azadiractha indica*			
	15 min	30 min	45 min	60 min	15 min	30 min	45 min	60 min
1.0	7.33±2.1^{a}	5.67±1.5^{a}	5.67±0.6^{a}	3.67±0.6^{a}	14.7±4.2^{a}	11.0±3.6^{a}	7.33±2.1^{a}	0.33±0.6^{a}
0.5	22.7±3.1^{b}	14.7±2.5^{b}	8.67±0.6^{a}	4.67±1.5^{a}	28.0±2.6^{b}	21.3±1.5^{b}	13.3±3.1^{b}	0.33±0.6^{a}
0.3	29.7±2.5^{c}	20.3±3.2^{c}	13.7±2.1^{b}	8.67±2.5^{b}	30.7±2.3^{b}	27.7±2.5^{c}	25.7±2.9^{c}	14.3±2.1^{b}
0.0 (control)	31.3±3.1^{c}	28.3±2.1^{d}	26.3±3.1^{c}	22.3±1.5^{c}	31.3±3.1^{b}	28.3±2.1^{c}	26.3±3.1^{c}	22.3±1.5^{c}
***p* value**	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*

Values are given as mean ± standard deviation. In each column, values with different superscripts have statistically significant different ($p< 0.05$)

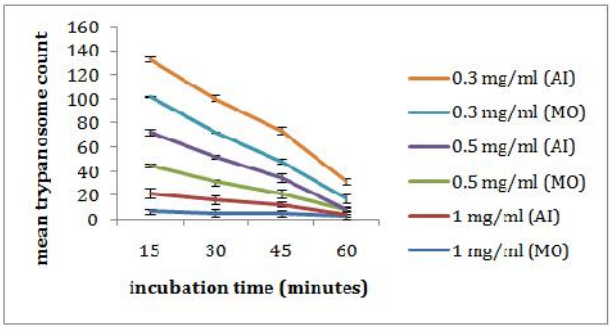

Fig 3: A chart showing the effect of three concentrations of ethyl acetate leaf extracts of *Moringa olifera* (MO) and *Azadiractha indica* (AI) on *Trypan brucei brucei* at four intervals of incubation. The effects of the extracts on trypanosome mortality increased with incubation time

4. DISCUSSION

The *in vitro* anti-trypanosomal activity of the ethyl acetate leaf extracts of *Moringa oleifera* and *Azadirachta indica* were separately evaluated and compared with each other. The results indicated that the solvent extracts from the leaves of the respective plants exhibited anti-trypanosomal activity compared to the controls. The results from this study thus collaborate those from previous studies [20].

The anti-trypanosomal effects exhibited by the extracts may be due to the presence of bioactive metabolites present in the plant extracts. Plants have long been proven to possess

chemical compounds with pharmacological and therapeutic properties. Ojiako [21] identified tannins, alkaloids, phenols, saponins and flavonoids [22] in the ethyl acetate leaf extract of *Moringa oleifera*. Alkaloids, flavonoids, saponins, phenols [23], sterols, and glycosides [15] were found in leaves of *Azadirachta indica*.

Comparisons of the anti-trypanosomal effect of the extracts indicated that the *Moringa oleifera* leaf extract exhibited higher anti-trypanosomal property than *Azadirachta indica* extract. At each time interval, it was observed that there was higher reduction in trypanosome counts in micro-titre wells containing *Moringa oleifera*. It would be expected that since the two extracts were prepared using the same solvent, ethyl acetate, they should contain the same bioactive principles and should have comparable effect on the test organism. The difference in the efficacy of extracts at the doges levels of the two plants may be due to the difference in the amount of secondary metabolites in each plant extractive more the secondary metabolites more the mortality of parasites. [21] stated that the major activity exhibited by a medicinal plant depends on the quantity of bioactive compounds it synthesizes. The percentage composition of the phytochemical constituents in *Moringa oleifera* has been shown to be significantly higher than the corresponding chemical entities present in *Azadirachta indica* [24,15].

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