Sahel Journal of Life Sciences FUDMA (SAJOLS)
December 2024 Vol. 2(4): 117-122
ISSN: 3027-0456 (Print)
ISSN: 1595-5915(Online)
DOI: https://doi.org/10.33003/sajols-2024-0204-16

Research Article

In Vitro Study of Ethanolic and Aqueous Leaf Extracts of *Azadirachta indica*, *Moringa oleifera* , and *Cymbopogon citratus* Against Diminazene Aceturate Resistance Strain of *Trypanosoma brucei brucei*

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ABSTRACT
African trypanosomiasis is a parasitic disease caused by arthropod vectors causing serious threats to the lives of millions of people as well as animals, especially cattle worldwide. Plants in Nigeria are known to contain medicinal properties within a large array of their chemical structures, and many have been screened and tested against various species of trypanosomes, in the effort to find new drugs against the disease caused by the resistance strain of *Trypanosoma brucei brucei* to diminazene aceturate (Berenil)®, ethanolic and aqueous leaf extracts of *Azadirachta indica, Moringa oleifera*, and *Cymbopogon citratus* were tested *in vitro* at varying concentrations. The ethanolic extract of *Azadirachta indica* was the most effective in terms of rapid motility cessation, with an impressive 7.5-minute cessation at the highest concentration (40 mg/ml). The aqueous extract of *Azadirachta indica* showed moderate activity at 40 mg/ml, but no effect at lower concentrations. The aqueous extracts of *Moringa oleifera* and *Cymbopogon citratus* showed minimal activity, with motility cessation only at the highest concentration in *M. oleifera* (10 minutes), while *C. citratus* exhibited no effect at any concentration. Furthermore, the ethanolic extract of *Moringa oleifera* showed no effect at any concentration, while diminazene aceturate (Berenil), despite being a standard treatment, showed no motility cessation within the 60 minutes in this experiment. *A. indica* ethanolic extract showed promising results as a potent alternative or adjunctive treatment for trypanosomiasis. This research indicates that the Nigerian plants may be suitable as a starting point in searching for novel and more efficient trypanocidal molecules.

Keywords: *Trypnasoma brucei brucei*; Phytochemicals; Toxicity; Resistance; *In vitro*; *In vivo*

Citation: Rabe, Y., Umar, Y.A., Ajibade, G.A. & Alhaji, A.I. (2024). *In Vitro* Study of Ethanolic and Aqueous Leaf Extracts of *Azadirachta indica*, *Moringa oleifera* , and *Cymbopogon citratus* Against Diminazene Aceturate Resistance Strain of *Trypanosoma brucei brucei*. *Sahel Journal of Life Sciences FUDMA,* 2(4): 117-122. DOI: https://doi.org/10.33003/sajols-2024-0204-16

INTRODUCTION

African trypanosomiasis is a protozoan parasitic disease of the genus *Trypanosoma*. *Trypanosoma congolense* (*T. congolense*), *Trypanosoma vivax* and *Trypanosoma brucei brucei* (*T. b. brucei*) are the species causing African Animal Trypanosomosis (AAT) which is referred to as *Nagana* in West Africa and *T. rhodesiense* and *T. b. gambiense* are responsible for sleeping sickness called Human African trypanosomiasis (HAT), while *T. evansi* and *T. equiperdum* cause Surra disease and dourine respectively. The disease is transmitted by a bite of the arthropod vector-tsetse fly (*Glossina* species) (Kelvin *et al.,* 2021). African Animal Trypanosomosis (AAT) is an enormous constraint to livestock and agricultural production in Tropical Africa, including Nigeria (Kelvin *et al.,* 2021).

Human African trypanosomiasis occurs in two forms, depending on the species of the parasites (and their subs-species), *Trypanosoma brucei gambiense*: in a species is found in 24 countries in Africa as well as central Africa. An individual can be infected with the disease without noticing, until after some weeks or months. Hence this disease account for almost 97% of sleeping sickness reported cases. The chronic stage result in the central nervous system being affected

(World Health Organization, fact sheets, 2020). *Trypanosoma brucei rhodesiense* is currently found in 13 countries in both eastern and southern Africa. Currently, this form represents less than 3% of reported cases and can advance to an acute infection. Signs and symptoms manifest weeks or months after infection. It develops faster and affects the central nervous system. The disease is only reported in Uganda.

In animal trypanosomiasis; Parasite species of the genus *Trypanosoma* are pathogenic to animals and cause disease such as *Nagana* in cattle, and leading to greater economic loss. Human pathogen especially *T. b. rhodesiense* parasites is also been harbored by domestic and wild animals serving as reservoir and they can as well act as a reservoir to *T. b. gambiense.* However, their specific role as a reservoir is not known (WHO, fact sheets 2020).

Due to toxic effects of conventional Drugs, parenteral administration, or are expensive and are not affordable by the farmers or the majority of the populace, and development of resistance mechanisms by trypanosomes towards these drugs currently being used. Despite the effort to eradicate the disease it has proven difficult to eradicate (Kelvin *et al.,* 2021). Thus, the search for medicinal plants with trypanocidal activities continue to generate a lot of research interest by the community of parasitologists. Although recent reports indicate antitrypanosomal activity exists in some medicinal plants, the potentials of many other plants used in folkloric medicine in Nigeria are yet to be investigated. This study is designed to investigate the in vitro and in vivo antitrypanosomal activity of selected plants in Kaduna Metropolis, Kaduna State North Western Nigeria. Findings of this study may provide alternative phytochemicals against the resistance strains of trypanosomes which will be safer, effective, cheaper and more accessible and can be applied externally or orally administered.

The aim of the study was to evaluate the anti-trypanosomal activity of extracts of *Azadirachta indica, Moringa oleifera,* and *Cymbopogon citratus* against dimenazene aceturate resistant strain of *T. brucei* in Wistar Mice In vitro.

MATERIALS AND METHODS

Source and Housing of Experimental animals

Four (4) Male and Female wistar mice aged 8–12weeks and weighing 25–35g were obtained from the National Institute for Trypanosomiasis, Research Kaduna, Nigeria.

Mice were housed in polypropylene cages with four mice per cage and allowed free access to clean water ad libitum and feed (pellet). All mice were kept at room temperature (23℃) and allowed for one week to acclimatize. Cages were cleaned and checked before introducing the mice.

Source of Parasites

T. *Brucei brucei* stabilates stored in liquid nitrogen were obtained from the National Institute for Trypanosomosis Research Kaduna, Nigeria. The parasites were maintained in the laboratory after inoculating in mice. Blood collected from donor mice at peak parasitaemia (10^7 parasites/ml of blood) was used for *in vitro* antitrypanosomal assay experiment (Nweze *et al.,* 2011).

Source, Collection and Identification of Plant

Azadirachta indica, Moringa Oleifera, and *Cymbopogon citratus* plants parts (leaves) were collected at different time within the environs of Kaduna, Kaduna state, Nigeria. Plants part collected were transported to the Laboratory of the Department of Biological Sciences, Nigerian Defence Academy, Kaduna. Plant parts were identified by a taxonomist in the Herbarium unit of Nigerian Defence Academy, Kaduna. Voucher number was assigned to each of the plant parts collected as follows: *Azadirachta indica* (NDA/BIOH/2365), *Moringa Oliefera* (NDA/BIOH/2366) and *Cymbopogon citratus* (NDA/BIOH/2367) and samples were deposited in the Herbarium unit of the Academy for reference.

Preparation of aqueous and ethanol leaf extracts

Plant parts (leaves) were washed and allowed to dry freely at room temperature (28^0C – 30^0C) for a period of one week, then grounded mechanically using electronic blender. The powdered materials were sieved and weighed before the procedures and stored in air-tight containers until required for use. The material in powdered form (100g) were soaked in 500ml of ethanol and aqueous and stirred intermittently for the period of 48 hours at room temperature (28^0C – 30^0C). The plant materials were extracted by maceration technique using 80% ethanol (LOBA CHEMIE PVT LTD) through mixing the grind and weighed plant material with 80% ethanol and aqueous in 1:7 ratios. After 72h maceration with regular shaking, the mixtures were strained using a strainer to remove solids and further filtered with sterile cotton wool and Whatsman filter paper No 1. The filtered solutions were evaporated using Rota vapour (BUCHI Rotavapor R-200) to remove the solvent to the acceptable level. Remnants from the Rota vapour was poured into Petri dishes and put in a dry oven at a temperature of 40 °C to remove the

remaining solvent. The prepared solid extracts were stored in a desiccator at 4^0C until needed (Debela *et al.,* 2020).

Laboratory Experiment

The experiment was conducted in the Biological Science Laboratory Nigerian Defence Academy and Botany laboratory Kaduna State University, Kaduna.

The *in vitro* antitrypanosomal activity of crude extracts

Different concentrations (2.5 - 40.0mg/ml) of crude extracts were prepared using phosphate buffered saline (PBS)/10% DMSO (dimethyl sulphoxide). Ten micolitres of each plant extract was mixed with 60µl of infected blood and the mixture incubated at 37^0 C for a period of 10 minutes in microtitre plate wells. For negative control, 10µl of extract Diminazene aceturate was replaced with PBS or 10% DMSO, Diminazene aceturate was used as positive control. After incubation, parasites were observed under the microscope (x 400) objectives for a cessation of motility every 5 minutes for a period of 60 minutes (Ayechew *et al.,* 2021).

Data Analysis

The data were carefully clean and checked to ensure correctness of entries before final analysis. The analysis was done using SPSS software version 26.0 (SPSS inc. Illinois, U.S.A., 2021). *P* values less than 0.05 were considered statistically significant (Feyera *et al.,* 2014).

RESULTS

Effects of graded concentrations of plant leaf extracts and diminazene aceturate on the time for cessation of *Trypanosoma brucei brucei* motility (in *vitro*)

The table presented below summarizes the effects of various plant extracts (leaf) and diminazene aceturate on the time taken for cessation of *Trypanosoma brucei brucei* motility. The graded concentrations of the leaf extracts and diminazene aceturate were administered to infected mice, and the cessation of trypanosome motility was observed over a set period. The data reveals a significant variation in the effectiveness of the extracts, with some demonstrating rapid motility cessation at higher concentrations, while others had no effect within the observation period (Table 4.1).

***Azadirachta indica* (Aqueous Extract)**: The aqueous extract of *Azadirachta indica* exhibited moderate efficacy in inhibiting trypanosome motility, as indicated by the time taken for motility cessation at different concentrations. At the highest concentration (40 mg/ml), motility cessation occurred after 55 minutes. However, as the concentration decreased, the time for cessation of motility increased. At 20 mg/ml and 10 mg/ml, cessation occurred at 60 minutes, which was the longest duration within the observation period. At lower concentrations (5 mg/ml, 2.5 mg/ml, and 0 mg/ml), no cessation of motility was observed, as denoted by the notation "> 60". This suggests that the aqueous extract of *A. indica* was only effective at the higher concentrations, with no observable effect at lower concentrations (Table 4.1).

***Azadirachta indica* (Ethanolic Extract)**: The ethanolic extract of *Azadirachta indica* demonstrated a highly potent effect at the highest concentration. At 40 mg/ml, motility cessation was observed after 7.5 minutes, indicating a rapid trypanocidal effect. However, at concentrations of 20 mg/ml and lower, no cessation of motility was recorded within 60 minutes (denoted by "> 60"). These findings suggest that the ethanolic extract of *A. indica* exhibits a dose-dependent effect, with significant activity only at higher concentrations (Table 4.1).

***Moringa oleifera* (Aqueous Extract)**: The aqueous extract of *Moringa oleifera* showed a moderate but dose-dependent response. At 40 mg/ml, motility cessation occurred after **10 minutes**, indicating that this extract also has some degree of trypanocidal activity at higher concentrations. However, at lower concentrations (20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 0 mg/ml), no motility cessation was observed within the 60-minute observation period, as all these values were marked as "> 60". This suggests that the aqueous extract of *M. oleifera* is effective at higher concentrations, but its efficacy diminishes significantly at lower doses (Table 4.1).

***Moringa oleifera* (Ethanolic Extract)**: The ethanolic extract of *Moringa oleifera* did not induce cessation of motility at any of the concentrations tested. At all concentrations (40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 0 mg/ml), no motility cessation was observed within the 60-minute period, as all results were recorded as "> 60". This indicates that the ethanolic extract of *M. oleifera* does not have an observable effect on trypanosome motility, even at higher concentrations (Table 4.1).

***Cymbopogon citratus* (Aqueous Extract)**: The aqueous extract of *Cymbopogon citratus* did not show any trypanocidal activity at any concentration tested. At all concentrations (40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 0 mg/ml), the cessation of motility occurred after "> 60" minutes, indicating no effect within the observation period. This suggests that the aqueous extract of *C. citratus*

has no significant effect on trypanosome motility, regardless of the concentration (Table 4.1).

***Cymbopogon citratus* (Ethanolic Extract)**: Similar to the aqueous extract, the ethanolic extract of *Cymbopogon citratus* also showed no effect on trypanosome motility at any concentration tested. Motility cessation was not observed within the 60-minute period at any of the concentrations (40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 0 mg/ml), as all results were recorded as "> 60". These findings suggest that neither the aqueous nor ethanolic extract of *C. citratus* has trypanocidal activity (Table 4.1).

Diminazene aceturate: Diminazene aceturate, the standard trypanocidal drug, did not induce cessation of motility of *T.brucei brucei* within the 60-minute observation period. At all concentrations (40 mg/ml, 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, and 0 mg/ml), motility cessation was recorded as "> 60" minutes. This suggests that the cessation of motility by diminazene aceturate might require a longer time frame than was observed in this experiment (Table 1).

Table 1: Effects of graded concentrations of plant extracts and diminazene aceturate on the time for cessation of *Trypanosoma brucei brucei* motility

Extract	Time (min) to cessation of motility					
	40 mg/ml	**20 mg/ml**	**10 mg/ml**	**5 mg/ml**	**2.5 mg/ml**	**0 mg/ml**
A. indica (Aq)	55	60	60	> 60	> 60	> 60
A. indica (EtOH)	7.5	> 60	> 60	> 60	> 60	> 60
M. oleifera (Aq)	10	> 60	> 60	> 60	> 60	> 60
M. oleifera (EtOH)	> 60	> 60	> 60	> 60	> 60	> 60
C. citrus (Aq)	> 60	> 60	> 60	> 60	> 60	> 60
C. citrus (EtOH)	> 60	> 60	> 60	> 60	> 60	> 60
Diminazene	> 60	> 60	> 60	> 60	> 60	> 60

KEY: *Azadirachta indica* Aqueous (*A. indica* Aq); *Azadirachta indica* Ethanolic (*A. indica* EtOH); *Moringa oleifera* Aqeous (*M. oleifera* Aq); *Moringa oleifera* Ethanolic (*M. oleifera* EtOH); *Cymbopogon citratu*s Aqeous (C. citratus Aq); *Cymbopogon citratus* Aqeous (C. citratus EtOH)

DISCUSSION

Studies on plant extracts have shown the importance of phytochemicals as possible sources of non-toxic, easily biodegradable substances and they may serve as precursors for the synthesis of useful drugs (Muhammad *et al.*, 2022).

On the *in vitro* culture, the resistance strain of *T. brucei* was introduced and the treatment commences, using the prepared plant extracts, *A. indica* was first introduced into the culture medium containing 96 microtitre plates' wells and left for 60 minutes at various doses, while monitoring for the parasites motility after incubating at 37^0c. the results shows that *A. indica* ethanolic is very effective in killing the parasites at lower dose of 40 mg/ml in 7.5 minutes by completely killing the parasites, confirming its effectiveness after the parasites motility was not observed during microscopy. Its counter parts which is aqueous kills the trypanosomes at 40mg/ml in 55 minutes. While *M. oliefera* aqueous *e*xtracts shows antitrypanosomal effects only at higher dose of 40mg/ml stops parasites motility, at 40mg/ml it was confirmed that the compound may not be toxic and cannot affect certain organs in animals at this dose, this was confirmed after the toxicity test was carried out as well as *in vivo* experiment. On the efficacy of *C. stratus* (aqueous and ethanolic) extracts tested in vitro, it has been observed that at both lower and upper dose (2.5-40mg/ml), *C. citratus* is not effective in clearing the parasites, but only slightly reduced the parasites loads, thus may be proving not possessing antitrypanosomal effects and lacking any potential use against resistance strain of *T.brucei brucei.*

Also. Ujah *et al.,* (2020) reported that *M oleifera* seeds extract showed high activity against *T.brucei,* at 10mg/ml within five minutes of incubation, the extracts eliminate all the parasites when tested in mice *in-vitro.* This is inline with our findings on *M. oleifera,* thou the plants parts used in our experiment on the in vitro assay differs, but our findings revealed the efficacy of *M. oleifera* (aqueous) leaf extracts in killing the trypanosomes at 40mg/ml in 55 minutes and have ability to stops parasites motility (thou not completely).

Aremu *et al.,* 2017, described *M. oleifera* as less efficacious against trypanasomes but could be co-administered with other drugs as a supportive. Because of its inability to treat *T. brucei* in mice even after 90hours treatment period. This is in contrast

with our findings on *M. olefera in vitro* assay showing some level of efficacy by the plant leaf extracts by killing the Trypanasomes at higher doses. The difference may b attributed to the solvents preparation, the type of solvents used, the measurement of the extracts and the location where the plant is collected as well as the strain of the Trypanasomes.

Report by Abubakar *et al.* (2019) in his research on the *in vitro* antitrypanosomal activities of Nigerian medicinal plants shows a fraction of *M. oleifera* leaves contain anti-trypanosomal activity at minimum inhibitory concentration of 25µmg/ml.This contradicts our findings showing *M. oleifera* efficacy in killing Trypanasomes at highest dose *in vitro*.

However, In vitro study of citral, the main component of *C. citratus,* revealed that the plant has antitrypanosomal activity (Cardoso *et al.,* 2010), in his study aimed at investigating both *in vitro* and *in vivo* anti-trypanosomal activities of polar (MeOH and aqueous) extracts of selected Meliaceae plant species against *T. b. rhodesiense*, *T. b. brucei* and *T. evansi*. Chromatographic fractionation of an active MeOH extract *A. indica* stem bark led to the identification of nimbin which was active against all the three trypanosome strains used in his study, this contradicts our findings on the *in vitro* assay of *C. citratus* leaf extracts showing inability of (both Aqeous and ethanolic) the extracts in killing the Trypanasomes.

When comparing the various plant extracts and diminazene aceturate, it is evident that the ethanolic extract of *A. indica* was the most effective in terms of rapid motility cessation, with an impressive 7.5-minute cessation at the highest concentration. The aqueous extract of *A. indica* showed moderate activity at 40 mg/ml, but no effect at lower concentrations. The aqueous extracts of *M. oleifera* and *C. citratus* showed minimal activity, with motility cessation only at the highest concentration in *M. oleifera* (10 minutes), while *C. citratus* exhibited no effect at any concentration. Furthermore, the ethanolic extract of *M. oleifera* showed no effect at any concentration, while diminazene aceturate, despite being a standard treatment, did not show motility cessation within the observed 60-minute period.

The data suggest that the ethanolic extract of *A. indica* possesses the strongest trypanocidal activity among the tested extracts, with rapid cessation of trypanosome motility at high concentrations. Other extracts, such as those from *M. oleifera* and *C. citratus*, demonstrated varying degrees of activity but were less effective than *A. indica* ethanol extract. Diminazene aceturate, while typically effective as a trypanocidal agent, did not show motility cessation within the 60-minute observation period in this experiment. Further studies with extended observation times or adjusted dosages may provide more insight into the time-course of diminazene aceturate's action. Overall, *A. indica* ethanolic extract shows promise as a potent alternative or adjunctive treatment for *T. brucei brucei*.

CONCLUSION

In conclusion, the data suggest that the ethanolic extract of *Azadirachta indica* possesses the strongest trypanocidal activity among the tested extracts, with rapid cessation of trypanosome motility at high concentrations. Other extracts, such as those from *Moringa oleifera* and *Cymbopogon citratus*, demonstrated varying degrees of activity but were less effective than *A. indica* ethanol extract. Diminazene aceturate, while typically effective as a trypanocidal agent, did not show motility cessation within the 60-minute observation period in this experiment. Further studies with extended observation times or adjusted dosages may provide more insight into the time-course of diminazene aceturate's action.

Conflict of Interest

No conflicting interest in this work

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