UNDER PEER REVIEW

Original Research Articles

Anti-Trypanosomal effect of Neem seed oil in mice infected with *Trypanosoma brucei Brucei.*

Abstract.

Aim: The anti-trypanosomal potential of neem seed oil was investigated in mice infected with *Trypanosoma brucei*.

Methodology: The mice were randomly divided into five groups containing 5 mice each(A,B,C and D). Group A was neither infected nor treated and served as control while groups B-D were intra-peritoneally infected with 1x 105 *trypanosoma brucei* organisms.

Results: The infected groups developed parasitaemia 3 to 4 days post infection(pi). The group that was untreated became ill and died 10 days post infection. The treatment with neem seed oil at the onset of infection(day 0) or onset of parasitaemia (day 4 pi) resulted in less severe clinical symptoms and reduced levels of parasitaemia. All the mice in both groups, infected/untreated and those treated with the oil(day 0 and 4 pi) died within 10mdays of the study period. Treatment with berenil cleared the parasites with all the mice surviving. The haematological indices of the berenil treated group showed significant($p<0.05$) recovery to almost their pre-infection levels.

Conclusion: The results suggest that although the neem seed oil did not have any significant effect on *Trypanosoma brucei* parasitaemia, it reduced the adverse haematological changes caused by the parasites. The results also suggest that the neem seed oil alone may not be useful in the control of *Trypanosoma brucei* infection.

Key words. Mice, Trypanosomiasis, neem seed oil, haematology.

1.INTRODUCTION.

African animal trypanosomosis is a parasitic disease common in both domestic and wide animals. It is caused by the organisms of the genus *Trypanosoma* [1]. It is one of the most economically important diseases in the sub-Saharan Africa [2]. It has been estimated that millions of cattle and people in this region are at the risk of contracting the disease from the 23 species and 33 sub species of tsetse flies [3]. There have been no drugs for the prevention and complete treatment of the disease [4-6].

The treatment of trypanosomosis is beset with difficulties including drug resistance [7], associated relapse parasitaemia [8], expensive nature of current trypanocides [2], and the side effects of current drugs [9]. The disease is endemic in Nigeria and the most commonly used chemical agent to control the disease is berenil (Diaminazene aceturate) [10].

UNDER PEER REVIEW

Azadirachta indica (Neem) has been utilized for ages as a viable source of traditional drugs [6] while the plant parts have been reported to have extensive medicinal properties [11]. The plant has also been reported to be used traditionally in the control of fungal [12], pests [13], bacterial [14], and viral infections [12]. Other reports show that the ethanolic leaf extract has trypanocidal potential [6, 15].

Because of the economic importance of the disease condition both in animals and humans coupled with the increasing incidences of drug resistance and lack of novel trypanocides, there is the need to search for cheaper and accessible trypanocides. Previous studies have focused on the effects of the methanolic and ethanolic extracts of *Azadirachta indica* on *Trypanosoma brucei brucei* [1, 6]. None of these have proved to be effective in the search for the treatment and control of trypanosome infection in animals. This study on the Neem seed oil is a further search for a trypanocide of plant origin. It is for these reasons that the study investigated the effects on the haematology of mice infected with the seed oil from *Azadirachta indica*.

2.Materials and methods.

2.1. *Trypanosoma Brucei* collection.

Trypanosome brucei (NITR strain 2295) parasites used in the study were collected from the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Plateau State, Nigeria. The parasites were previously isolated from cattle on the Jos Plateau, Nigeria and were stabilized by four passages in rats before storage in liquid Nitrogen. The stabilate was passed twice in rats before use in the study. The parasites were transported in rats and mice to Veterinary Pathology department University of Maiduguri, Borno State Nigeria.

2.2. Collection of Neem seeds and extraction of the seed oil.

Fresh ripe Neem seeds were collected from the Neem trees planted within the University campus. They were washed under tap water and the dried seeds peeled off to remove the hard shell leaving the kernel. The kernels were air dried under room temperature and kept in a clean container for the oil extraction. The Neem seed oil was extracted by the traditional methods of extraction. The seed kernels were grounded to a powder form with the aid of a pestle and a mortar. Appropriate amount of distilled water was added to the dry ground kernel powder in a clean container and a little pressure was applied to the mixture to squeeze out the oil from the kernel. At the end of the process about 40ml of light brown virgin Neem seed oil was extracted from 24.3kg of the kernel seeds.

2.3. Animal treatment.

The animals used in the study were adult tropical breed albino mice that weighed between 21.8-37.9g and were twenty in number. They were obtained from the Immunology department of the University of Maiduguri teaching hospital and transported to the veterinary pathology

UNDER PEER REVIEW

department of the University for the study. The animals were kept in rat cages and feed animal feed (growers' marsh, Buruku Mills Jos) and water provided *ad libitum*. The animals were acclimatized for one week before the start of the Experiment. Ethical approval was gotten from the Experimental Animal Ethics Committee(EAEC) of the College of Medical Sciences of the University. Thereafter, the animals were randomly divided into five groups(A, B, C, D, and E) of five mice each. The mice in group A (control) were neither infected with the parasites nor treated with the seed oil or drug while groups B, C, D and E were inoculated by a single intra-peritoneal injection with 0.5ml of infected rat blood containing 1X105 trypanosomes. Animals in group B were injected intra-peritonealy with 10mg/kg body weight of the Neem seed oil extract *ad libitum* while group C were also injected with 10mg/kg body weight of the Neem seed oil at the onset of parasitemia. The animals in group D were treated once intramuscularly with 3.5 mg/kg body weight Berenil (Diminazene aceturate, Farbwerke, AG, Frankfurt, Germany). The animals in group E were infected but remained untreated (positive control).

2.4. Estimation of parasitaemia and haematological parameters.

The packed cell volume (PCV) was done by the microhaematocrit method, while the erythrocyte red blood cell (RBC) count and the haemoglobin estimation (Hb) were as referenced by Schalm *et al.* [16]. The haematological parameters were noted every day following treatments and on day **5** post infection when parasitaemia was first observed. The initial observation of parasitaemia was done by the wet microscopy and buffy coat methods as earlier described [10, 17]. The extent of parasitaemia was evaluated using the matching method described by Herbert and Lumsden [18]. All the experimental animals were carefully observed daily for symptoms of trypanosomiasis.

2.5. Statistical analysis. Data obtained were presented as graph for the packed cell volume and as means in table for the parasite count. The data were analyzed using ANNOVA followed by the student t-test. The acceptable level of significance was $P<0.05$.

3. Results.

3.1. Clinical manifestations.

The animals in the infected groups (B, C, D and E) showed clinical symptoms of trypanosomiasis with varying degrees of lethargy, pale mucus membranes, rough hair coats and reduced feed intake. The disease progressed in all the infected groups but was most severe in the infected but untreated group (Group E). The mice in uninfected group (control) were healthy and active, and showed good appetite.

3.2. Parasitaemia.

The results of the parasitaemia on the infected groups (B, C, D and E) are represented in Table1. The results showed that there was an increased parasitaemia that remained high in the infected

UNDER PEER REVIEW

groups. Parasites were first detected in the circulation of all the infected groups by the second and fourth days post infection. Thereafter parasitaemia rose steadily in the infected groups until the death of all the mice in the groups. There was no significant difference between the infected but treated groups (groups B and C) and infected but untreated group (group E) although parasite count was slightly lower in the treated groups. The results also showed that in the group treated with the drug berenil (group D), that no trace of trypanosome was seen in circulation two days post infection and remained so throughout the study.

3.3. Haematology.

The results of the haematological observations are shown in figures 1, 2, and 3 respectively. Following infection, the packed cell volume (PCV) of all the infected groups decreased to lower values following the onset of parasitaemia which continued to their lowest values by day 8 post infection. A similar trend was noted in the other groups (B, C) treated with the extract. The decrease in PCV was sustained in group E that was infected but untreated until the death of all the mice. On the other hand, treatment with berenil resulted in the recovery of PCV towards the normal pre-infection values from day six post infection. This was maintained to the end of the study. The control group did not show any significant change in the PCV values during the study (Figure1).

The haemoglobin (Hb) concentration dropped sharply and continued until all the animals in the untreated group died. In the groups treated with the extract the Hb concentration reduced from day 4 post infection and this was sustained to the end of the study. The reduction in the Hb concentration was higher in the untreated group than the extract administered groups. In the group treated with berenil, the Hb concentration was almost recovered to the pre-infection values day 6 post infection(Figure 2). The result of the red blood cell (RBC) count fluctuated but indicated similar trends to those of PCV and Hb in all the groups throughout the study period (Fig.3).

Table 1. Parasitaemia determination.

Days post infection	Group A	Group B	Group C	Group D	Group E
0	0	0	0	0	0
2	0	6.1	6.1	0	6.1
4	0	8.2	8.6	0	8.1
6	0	8.7	8.7	0	8.9
8	0	9.0	9.0	0	9.0

Values are represented as log no of parasite.

UNDER PEER REVIEW

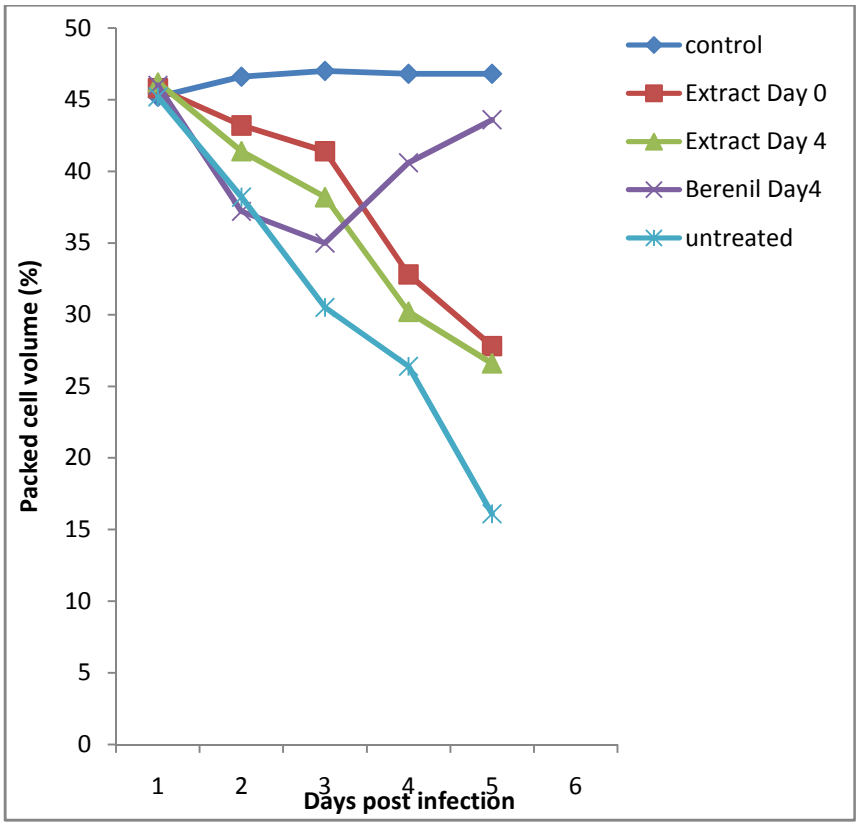
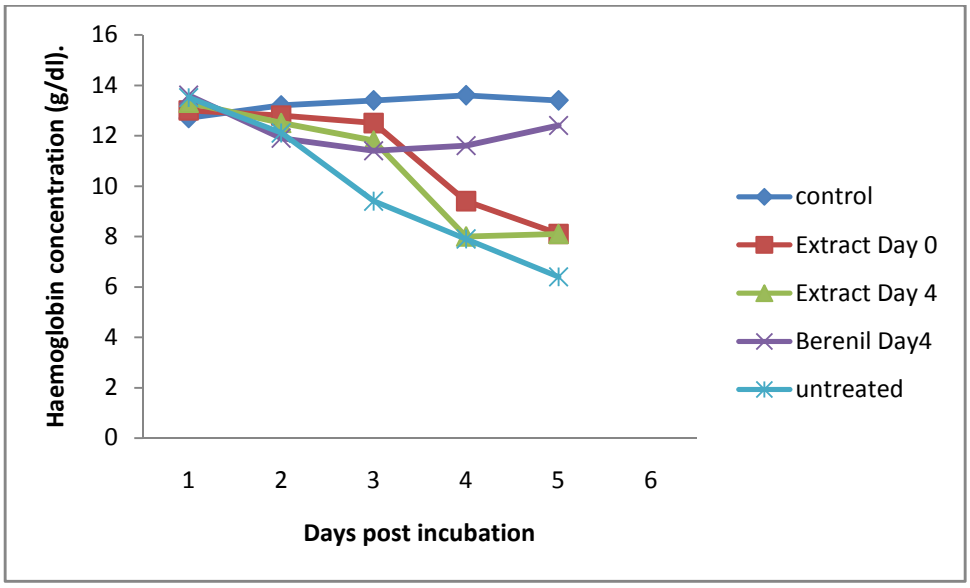


Figure1. Packed cell volume (PCV) (%) of the mice infected with *Trypanosome brucei*.



UNDER PEER REVIEW

Figure 2. Haemoglobin concentration (g/dl) of the mice infected with *Trypanosome brucei*.

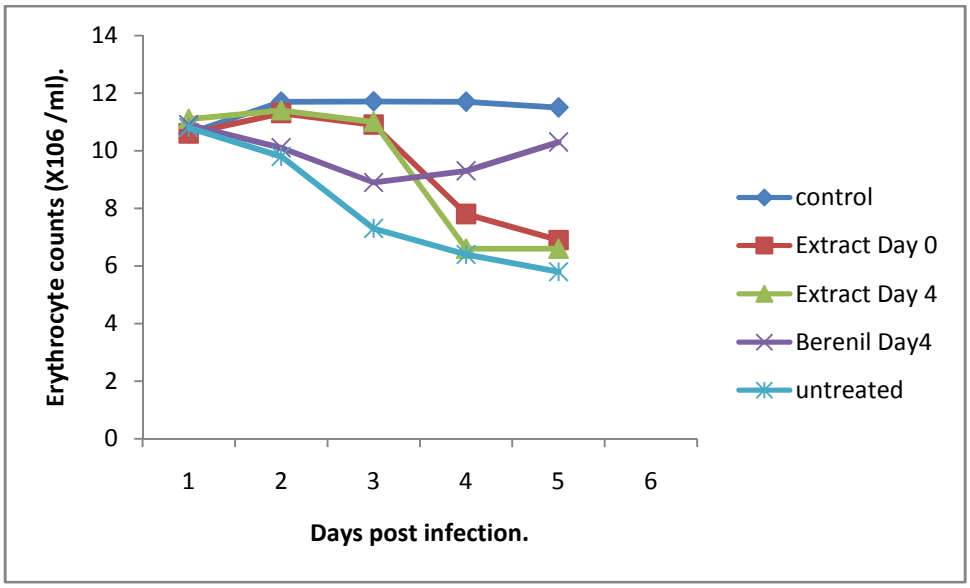


Figure 3. Red blood cell count (mm^3) of the mice infected with *Trypanosome brucei*.

4. Discussion.

The results from the study indicated that the treatment dose of the neem seed oil used was not effective in clearing *Trypanosoma brucei* from the blood of the infected mice. This can be seen from the continued detection of the parasites in the blood of the infected mice throughout the study period. Salivarian trypanosomes are characteristically known to exhibit antigenic variations that may be responsible for the waves of successive parasitaemia observed during their infections [10]. The outcome of the disease is dependent on the ability of the host to limit the peak of parasitaemia and even eliminate infection [19]. It must be observed that the genetic makeup of trypanosome population is a very important factor influenced by the transmission intensity which in turns may determine the rate of drug resistance [1].

We observed from the results that irrespective of the susceptibility of the host when administered equal dose of the parasites that the initial rate of replication were similar in all the infected groups. This is in line with results of Nwosu *et al*. [10]. This could explain the increased parasitaemia in all the infected groups (BCE) after the initial detection of parasites in the blood and its progression during the study period. The results of Nok *et al*. [6] showed that the leaf

UNDER PEER REVIEW

extract of *Azardirchta indica* has trypanocidal effect against *Trypanosoma brucei* while the results of Omoja *et al.* [1] showed that the combination of the leaf extract and diminazene diaceturate was more effective in the treatment of trypanosomosis caused by *Trypanosoma brucei.*

The level of anaemia was determined by the estimation of the PCV, Hb and RBC respectively. It has been reported previously that anaemia in trypanosomosis starts during the first wave of parasitaemia [19, 20]. There was a steady decline in the haematological profile of the infected groups (groups B,C) treated with the Neem seed oil which was most severe in the infected but untreated group(group E). Although, there was a decline in the haematology, the seed oil also led to the slight recovery in the haematological parameters of the infected groups thus suggesting a modulatory effect. However, the results showed that there was a significant recovery of the haematological profile of the group administered with berenil when compared with the other infected groups. Also, the results showed that treatment with berenil cleared the parasites from circulation two days after infection. The results of Nwosu *et al.* [10] showed that the stem bark extract of *Sacoglottis gabonensis* improved the haematological profile of rats infected with *Trypanosome congolense*. Anaemia has been shown in previous reports to be common in natural and experimental trypanosomosis in animals [10, 19, 21-25].

Because of the shorter period of observation and the single dose administration, we could not determine if there was relapsed infection in the group treated with berenil as relapse infection has been reported in some previous studies following treatment of trypanosome-infected animals [1, 26-28]. This could be because of the inability of the drug to cross the blood-brain barrier [29,30].

In conclusion, the seed oil of *Azadrichta indica* does not possess tryponocidal activity against *Trypanosoma brucei brucei* as against the reported anti-trypanosomal activity of the leaf extract. Also, the seed oil has modulatory effect on the haematology of the infected mice.

References.

1. Omoja VU, Anaga AO, Obidike IR, Ihedioha TE, Umeakuana PU, Mhomga LI, et al. The effects of combination of methanolic leaf extract of *Azadirachta indica* and diminazene diaceturate in the treatment of experimental *Trypanosoma brucei brucei* infection in rats. Asian Pacif J Tropic Med. 2011; 337-341.
2. Holmes PH, Eisler MC, Geerts S. Current chemotherapy of animal trypanosomiasis. In: MaudlinI, Holmes PH, Miles MA, editors. The Trypanosomiasis. Wallingford: CAB International; 2004
3. Samdi SM, Abenga JN, Attahir A, Wayo BM, Sumayin HM, Haruna MK, et al. Constrains in the control of African Trypanosomiasis: the prevailing factor in Kurmin Kadun, Northen, Nigeria. Inter J Anim. Vet. Adv. 2010; 2(1):31-36.

UNDER PEER REVIEW

4. Decamp R, Moreno SNJ. Free radical metabolites in the mode of action of chemotherapeutic agents and phagocytic cells in *Trypanosoma cruzi*. Rev. Infect. Dis. 1984; 6:223-228.
5. Nicholas J, Murgolo AC, Graeme BH. Trypanothione reductase. Ann. NY Acad. Sci. 1989; 569:193-218.
6. Nok AJ, Esievo KAN, Longdet I, Arowosafe S, Onyenekwe PC, Gimba CE, et al. Trypanocidal potentials of *Azadirachta indica*: In Vivo activity of leaf extract against *Trypanosoma brucei brucei*. J. Cli. Biochem. Nutr. 1993; 15:113-118.
7. Geerts S, Holmes PH. Drug management and parasite clearance in bovine trypanosomiasis in Africa. The programme against African trypanosomiasis (PAAT), technical and scientific series I. Rome: FAO; 1998; pp. 5-31.
8. Egbe-Nwiyi TN, Igbokwe IO, Onyeyili PA. Relapse of infection in single and mixed trypanosome infections in rats after Diminazene aceturate treatment. Vertiarski Arhiv. 2006; 76(3):255-262.
9. Asuzu IU, Chineme CN. Effect of *Morinda lucida* leaf extract on *Trypanosoma brucei* infection in mice. J Ethnopharmacol. 1990; 30:307-313.
10. Nwosu CO, Maduka HCC, Mahe A, Adamu M, Nwagbara ND. Reduction of the effects on the haematology of rats infected with *Trypanosoma congolense* by ethanolic extract of *Sacoglottis gabonensis* stem bark. Nig J Botany. 2010; 23(1):41-54.
11. Okpanyi SN, Ezenkwu GC. Anti-inflammatory and anti-pyretic activities of *Azadirachta indica*. Planta Med. 1981; 4:44-45.
12. Sai Ram M, Liavazhagan G, Sharma SK. Antimicrobial activity of a new vaginal contraceptive: NIM-76 from neem oil. J Ethnopharmacol. 2000;71: 377-382.
13. Schmutterer H. Properties and potential of natural pesticides from the neem (*Azadirachta indica*). Annu. Rev. Entomol. 1990; 35:271-297.
14. Okemo PO, Mwatha WE, Chhabra SC, Fabry W. The kinetics of *Azadirachta indica, A. juss (Meliaceae)* extracts on *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans*. Afri J Sci Technol. 2001; 2:113-118.
15. Hanifah AL, Awang SZ, Ming HZ, Adidin SZ, Omar MH. Acaricidal activity of *Cymbopogon citrates* and *Azadirachta indica* against house dust mites. Asian Pacif J Tropic Biomed. 2001; 1(5):365-369.
16. Schalm OW, Jain NC, Carrol EJ. Veterinary Haematology. 3rd edn., Lea and Febiger, Philadelphia; 1995.
17. Murray M, Trail JCM, Tuner DA, Wissoq Y. Livestock productivity and trypanotolerance. Network Training Manual, ILCA, Addis Ababa, Ethiopia; 1983.
18. Herbert WJ, Lumsden WHR. Trypanosome brucei: a rapid “matching” method for estimating the host’s parasitaemia. Exp Parasitol. 1976; 40:427-431.
19. Nwosu CO, Ikeme MM. Parasitaemia and clinical manifestations in *Trypanosoma brucei* infected dogs. Revue d’Elevage eet de Medecine Veterinare des pays Tropicaux, 1992; 45:273-277.

UNDER PEER REVIEW

20. Anosa VO. Studies of the parasitaemia, plasma volumes, leucocytes and bone marrow cell counts and moribund state in Trypanosoma brucei infection of spleenectomised and intact mice. Zentbl. Vetmed. 1980; 27:169-180.
21. Anosa VO. Experimental Trypanosom vivax infection in sheep and goat: the relationship between parasitaemia, the growth rate and the anaemia. J Nig Vet Med Ass. 1974; 3:102-108.
22. Anosa VO. Mammalian blood cells in health and trypanosomiasis. Tropic Vet. 1983a; 1: 177-199.
23. Anosa VO. Diseases produced by *T.vivax* in ruminants, horses and rodents. Zentbl. Vet. Med. 1983b; 30:717-741.
24. Anosa VO. Haematological and biochemical changes in human and animal trypanosmosis. Part I. Revue d' Elevage et de Medecine Veterinaire des pays Tropicaux. 1988a; 41:65-78.
25. Anosa VO. Haematological and biochemical changes in human and animal trypanosmosis. Part II. Revue d' Elevage et de Medecine Veterinaire des pays Tropicaux.1988b; 41:151-164.
26. Morrison WI, Murray M, Whitelaw DD, Mahmood M. Pathology of infection with *Trypanosoma brucei*: Disease syndromes in dogs and cattle resulting from severe tissue damage. In: From parasitic infection to parasitic disease. Contr Microbiol Immunol. 1983; 7:103.
27. Moulton M, Trail JCM, Turner DA, Wissoq Y. Relapse infection after chemotherapy in goats experimentally infected with *Trypanosoma brucei*: pathological changes in central nervous system. Veterinary Pathology. 1986; 23:21-28.
28. Rabo JS, Wakil AB, Maduka HCC. Effects of Berenil on the pathogenesis of trypanosomiasis in *Trypanosoma brucei* infected rats. Nig J Ani Prod. 1994; 21: 142-148.
29. Jennings FW, Gray FD, Urquhart GM. (1982). The use of Erlangen dimidine 98/202 in relapsing *Trypanosoma brucei* infections in mice. Transact Royal Soc Tropic Med Hyg. 1982;76:204.
30. Rouse BT, Kennell SJ, Huang L. Drug delivery by immunoliposomes. In: Chemotherapy for Trypanosomiasis. Proceedings of a Workshop Held at ILRAD Nairoby, Kenya, 23-24 August. 1989; Pp.115-118.