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Anti-trypanosomal effects of *Azadiracta indica* (neem) extract on *Trypanosoma brucei rhodesiense*-infected mice

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Abstract. An *in vivo* study was carried out to determine the anti-typanosomal effect of aqueous extracts of the bark of *Azadiracta indica* (neem) in *Trypanosoma brucei rhodesiense*-infected mice. The extracts were orally administered *ad libitum* twenty four hours post-infection for three days to mice after dose determination and toxicity testing. The effect of the extracts in trypanosome-infected mice was monitored for 20 days by determining changes in the packed cell volume (PCV), parasitemia levels and survival rate. The bark extracts of the neem plant did not show any acute toxicity to the uninfected animals because no significant effect on weight and PCV was recorded. However, infection with *T. b. rhodesiense* led to a decrease in weight and PCV, the decrease being more in those animals that were given water only and low doses of plant extracts. The extracts produced a dose-dependent effect at delaying onset of parasites appearance in circulation, decreasing level of parasitemia and PCV. Treatment with 1000 mg/kg of plant extract was comparable to and in some cases more effective than suramin, a known trypanocidal drug.

Key words: Azadiracta indica, trypanosomosis, parasitemia, packed cell volume, survival rate

1. Introduction

Trypanosomiasis is an important protozoan disease of domestic animals and man. Human African trypanosomosis (HAT) is caused by the tsetse fly-transmitted hemo-flagellates *Trypanosoma brucei rhodesiense* (in East and Southern Africa) and *T. b. gambiense* (in West and Central Africa), while animal trypanosomosis is caused by *T. b. brucei*, *T. vivax* and *T. congolense* (1). Sleeping sickness has been on the rise in recent years and is viewed as a major health problem in many African countries, with sixty million people being at risk of infection in sub-Saharan Africa (2).

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Trypanosomes have a glycoprotein coat that is encoded by genes that are antigenically distinct thus making the parasite able to engage an immune-evasive process of antigenic variation (3). Due to this process, the prospects for vaccine development are poor. Drugs are the only viable management options for tackling this problem. However, all the currently available drugs have serious limitations, which include high cost, serious side effects, long-course of parenteral administration, variable efficacy and emergence of drug resistant trypanosome strains (4-8). The presence of drug resistant trypanosomes has recently risen to alarming proportions (7-10). Treatment of the late stage of sleeping sickness with Melarsoprol (Mel B), a trivalent arsenical, is hazardous, causing reactive encephalopathy in 5-10% of patients treated, with 1-5% mortality (11). The problem of drug resistance has been aggravated by lack of new drug development initiatives by major pharmaceutical firms. There is therefore, an urgent need to develop new effective and safe chemotherapeutic agents for the treatment of African sleeping sickness.

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The natural world has over the years been a major source of medicinal agents and despite the recent advances in pharmacology and synthetic organic chemistry, plant biomolecules (phyto-compounds) continue to provide key lead structures and therapeutic agents for the treatment of protozoan diseases (12,13) with approximately 20,000 species of higher plants being used medicinally throughout the world (14). The use of herbal remedies in the treatment of trypanosomosis is potentially promising with some ethnomedicinal plants used against the diseases having been demonstrated to be potent trypanocides (15-17). Pharmacologically active compounds of plant origin can provide an alternative to chemically synthesized drugs to which many infectious microorganisms have become resistant (18). The stem bark extract of *Ximenia americana* has shown *in vitro* anti-trypanosomal activity against *Trypanosoma congolense* (8). Furthermore, several well-known and efficacious anti-malarial drugs such as quinine and artemisinin have their origin in plants (13, 14, 19). *Azadirachta indica* (neem plant) has been recognized for thousands of years especially among traditional Indians as a medicinal plant. The biological activities of some of the phyto-compounds in the neem plant have recently been reviewed (20). Oil from the neem leaves, seeds and bark exhibit a wide spectrum of anti-bacterial action (21). The oil has also been reported to have some anti-protozoan properties (22). In spite of this knowledge, very little work has been carried out to establish the *in vivo* anti-trypanosomal activity of neem extracts and none with the human infective *Trypanosoma b. rhodensiense*. This study was therefore aimed at investigating the *in vivo* anti-trypanosomal activity of crude extracts from the bark of *Azadirachta indica* in mice infected with *T. b. rhodesiense*.

2. Materials and methods

2.1. Plant materials

Plant materials (bark) of *Azadirachta indica* were collected from Kisii town, west of the Great Rift Valley in Kenya. After identification by a plant taxonomist, a reference specimen was collected and deposited at the department of Biological Sciences, Egerton University.

2.2. Sample preparation

The bark of the plant was thoroughly washed and dried at room temperature to a constant weight. The dried bark was milled to fine powder and then stored in a dry container.

2.3. Extraction and concentration of crude extract

Thirty grams (30g) of powdered sample were soaked in 300ml of 100% dichloromethane for five days away from direct light. The supernatant was decanted and filtered using Whatman filter (No. 54). The filtrate was evaporated to dryness *in vacuo* using a rotary evaporator to concentrate it. The sample yielded was stored in dark brown bottles at 4 ^{0}C to avoid biological degradation.

2.4. Experimental animals

All experimental protocols and procedures used on animals during the study were reviewed and approved by the institutional animal care and use committee (IACUC).

A standard protocol was drawn up in accordance with the Good Laboratory Practice (GLP) regulation of the World Health Organization (2). Randomly selected male Swiss white mice 6-8 weeks old and weighing between 20-25g were housed in standard mice cages in a controlled environment and provided *ad libitum* access to food and water with or without different concentrations of neem extracts.

2.5. Trypanosomes and infection

Cryopreserved *Trypanosoma brucei rhodesiense* isolate (KETRI 3798) was obtained from Trypanosomiasis Research Centre (TRC) trypanosome bank. The parasites were propagated and maintained in clean Swiss white mice few days before the commencement of the research.

2.6. In vivo toxicity test

Male Swiss white mice (20 to 25g) all from the TRC colony were used. The mice were brought to the experimental laboratory for acclimatization for 7 days pre-treatment with test extracts. Twenty mice were thereafter randomly grouped into four groups of five animals per group, with each group receiving the following concentration of daily oral neem bark extract treatment for 14 days: Group i (250mg/kg body weight (bwt); Group ii (500mg/kg bwt); and Group iii (1000mg/kg bwt). Group iv was given water only. The mice were then closely monitored throughout the 18 days of administration for overt toxic response. Animals were initially closely observed for a period 10-15 min following administration of the test drug for signs of acute toxicity, including the hypotensive response (dyspnea and

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lethargy) elicited by test compound at its various doses. The overall health and general well-being of the treated mice was observed and recorded on a daily basis for the rest of the experimental period. Excessive weight loss and changes of packed cell volume of more than two-fold compared with the water treated control group of mice over the 2 week dosing period was considered a key indicator of declining health due to drug toxicity.

2.7. In vivo efficacy determination of infected experimental animals

Male Swiss white mice (20 to 25g) from the TRC colony were used. Thirty mice were randomly divided into six groups of 5 mice each. The mice were brought to the experimental laboratory for acclimatization for 14 days. All the mice were then intra-peritoneally infected with 1 x 10^5 trypanosomes of *T. b. rhodesiense* (KETRI 3798) from donor mice from the Protozoology Division of TRC.The mice were then treated as follows: Group I; *ad libitum* oral treatment of 250mg/kg bwt of extract, 24 hours post-infection (PI) for three days; Group ii ; *ad-libitum* oral treatment with 500mg/kg bwt of extract from 24 hours PI for 3 days; Group iii; *ad-libitum* oral treatment with 1000mg/kg bwt of extract from 24 hours PI for 3 days; Group iv; infected and given water *ad-libitum* with no extract (infected control); Group v; intra-peritoneally treated using Melasorprol (Mel B) (1mg/kg bwt) once at 24 hours PI (positive control); and Group vi- intraperitoneally treated using Suramin (5mg/kg bwt) once at 24 hours PI (positive control). The *in vivo* efficacy was determined based on the changes in the levels of parasitemia, packed cell volume (PCV) and weights of the animals during the experimental period. The Kaplan Mayer curve was drawn to compare the effects of drugs on the survival time of the animals during the experimental period.

2.8. Parasitemia, blood sampling and determination of packed cell volume

The parasitaemia level for each mouse in every group was determined at two days interval. To estimate the number of circulating parasites in infected mice, two methods were used: the rapid “matching” method by Herbert and Lumsden (22) when parasite were seen by direct microscopy and the buffy coat technique as described by Murray *et al.* (23) when parasites could not be seen by direct microscopy. At two days interval, blood was taken from each mouse by tail snip into 100µl microhaematocrit tubes for PCV determination by the method by Woo (24). The average for each group was then calculated for each sampling point.

2.9. Effect of extracts on survival of trypanosome infected mice

The average survival rates of individual mice were calculated after recording of specific dates of death for each mouse post-infection and compilation of data done for each treatment group.

2.10. Statistical analysis

Data was analyzed using Statsview® Statistical programme (SAS) and the means for PCV, parasitaemia and weights changes were compared using the student t-test. Univariate survival analysis of data using Kaplan-Meier method was done to determine the effect of neem on the survival rate of infected animals. The log-rank test was used to examine the null hypothesis that the survival curves were identical.

3. Results

3.1. Toxicity test in uninfected mice

Dosages of 250, 500 and 1000mg/kg bwt of the neem extracts did not significantly affect the levels of packed cell volume ($p>0.05$) (Fig. 1) and weight ($p>0.05$) (Fig. 2) of the uninfected mice throughout the experimental period when compared to those of water- treated mice. All the mice showed a gradual increase in body weight and PCV during the toxicity test period. The results show that the plant extract was not toxic to the treated mice during the experimental period.

3.2. Parasitaemia in trypanosome infected mice

There were significant differences in the parasitaemia levels between the various drug

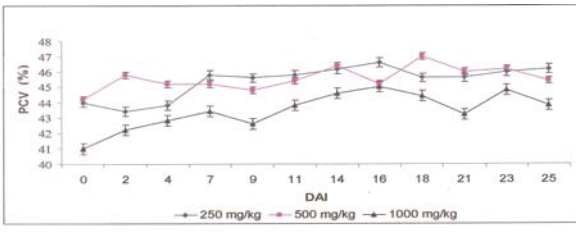

Fig. 1. PCVs of uninfected mice during toxicity testing. The uninfected mice were treated *ad libitum* with either neem extracts at 250, 500, or 1000mg/kg bwt, or water only (the controls). No significant differences were observed between the controls and the treated animals ($p>0.05$).

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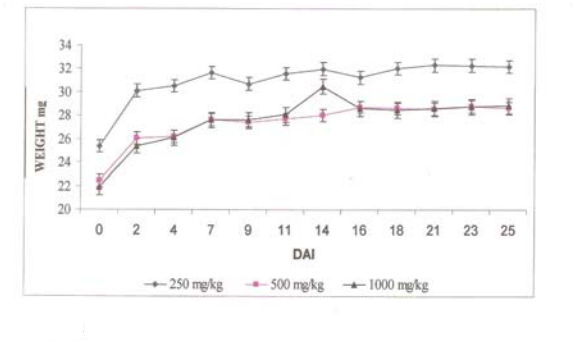

Fig. 2. Weights of uninfected white mice during toxicity testing. The mice were given neem plant extracts *ad libitum* at 250, 500, or 1000mg/kg bwt. The mice were weighed daily for 12 days.

treatments during the infection (Fig. 3). Water-treated mice showed presence of parasites 4 days after infection (DAI) with all the mice being positive by 7 DAI. There was a delay in the commencement of parasite appearance in blood of infected mice that were treated with neem plant extract and suramin. The time delay in parasite appearance in neem treated mice was dose dependent. The times of parasite appearance were as follows: 250mg/kg bwt treatment group (positive at 7 DAI and all positive by 9 DAI); 500mg/kg bwt treatment group (positive at 9 DAI and all positive by 11 DAI); 1000mg/kg bwt treatment group (positive at 11 DAI and all positive by 13 DAI).

The Mel B-treated mice did not show any parasites at any stage of experiment while suramin-treated mice showed parasites 4 DAI with all the mice being positive by 11 DAI. Comparison of the parasitaemia levels in various treatment groups at 7 and 9 DAI when all mice for each group were still alive showed that mice treated with water and 250mg/kg bwt neem extract had the highest level of parasites. The higher neem extract concentrations showed a dose-dependent effect on the level of parasitemia. Indeed, the 1000mg/kg neem extract treatment was comparable to the suramin positive control at controlling parasitaemia levels.

3.3. Packed cell volume in trypanosome infected mice

The changes in the PCV levels for the various treatment groups are presented in (Fig. 4). There was a gradual fall in the mean PCV levels starting 2 DAI in all infected mice. However, there was a significant improvement in decrease in the drop in the PCV levels that was dose-dependent in the neem plant extract-treated mice ($p<0.05$). The 250mg/kg bwt neem extract treatment group showed the highest and fastest drop in PCV, comparable to the water-treated group of mice. The decline in PCV levels was lower in the 500mg/kg bwt treatment group and even less in the 1000mg/kg bwt group. The 1000mg/kg bwt treatment and suramin were comparable in reducing the drop in PCV. However, the Mel B-treated mice did not show any significant drop in the PCV during the infection period ($p>0.05$).

3.4. Weight changes of trypanosome infected mice

All trypanosome-infected mice except those treated with Mel-B showed a significant decline in body weight during the experimental period (Fig. 5). The water, 250 and 500mg/kg bwt treatment groups had the most significant decline in body weight ($p<0.05$). The 1000mg/kg bwt neem extract- and suramin-treated groups showed comparable but significant decline in body weight that was less drastic than in the other groups. However, the Mel-B treated group showed a gradual but significant increase in body weight, especially towards the end of the experimental period ($p<0.05$).

3.5. Survival periods of trypanosome infected mice

The survival periods are as shown in (Fig. 6). There were significant differences in the time points in the death of mice following the infection. Deaths in water-treated mice started 4 DAI with all the mice dying by 7 DAI. There was an improvement in the survival of infected mice that was dose-dependent. With 250mg/kg bwt, deaths commenced at 7 DAI and all died by 9 DAI while with 500gm/kg bwt deaths commenced at 9 DAI and all died by 11 DAI. In the 1000mg/kg bwt treatment group, death commenced at 11 DAI and all died by 13 DAI, while the survival of suramin-treated group was comparable to that of 1000mg/kg bwt-treated mice. The Mel B- treated mice on the other hand did not show parasitemia at any stage of experiment and thus survived to the end of the experimental period.

4. Discussion

Results from the study indicate that neem extract even at 1000mg/kg bwt did not have toxic effects to mice as measured by changes in body weight and PCV. This supports evidence reported elsewhere that neem bark extract has no toxicity

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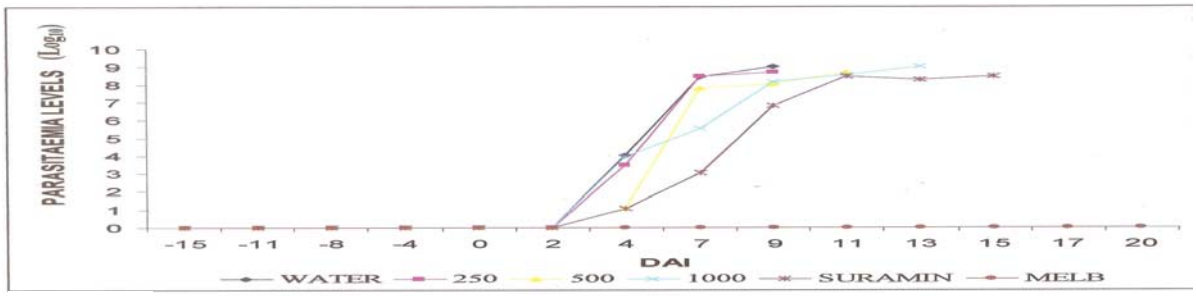

Fig. 3. Parasitemia of *T. b. rhodesiense* infected mice treated with either Mel B, suramin, neem extract at 250, 500 or 1000mg/kg bwt, or water only. Only Mel B, suramin and neem extract at 1000mg/kg bwt were effective in controlling the parasitemia.

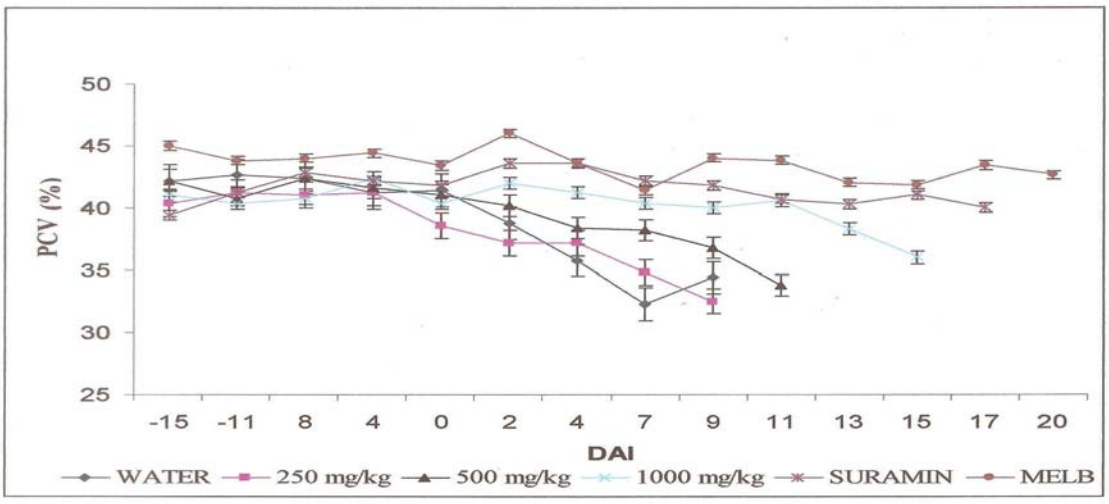

Fig. 4. PCVs of *T. b. rhodesiense* infected mice treated with either Mel B, suramin, neem extract at 250, 500, or 1000mg/kg bwt, or water only. Animals treated with either Mel B, suramin or neem extract at 1000mg/kg bwt had significantly higher PCVs levels than the other 3 groups ($p>0.05$).

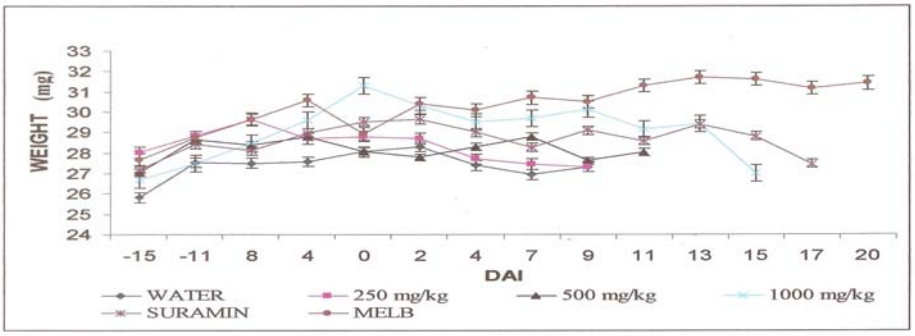

Fig. 5. Weights of *T. b. rhodesiense* infected mice treated 24 hours later with either Mel B, suramin, neem extract at 250, 500 or 1000mg/kg bwt, or water only. Mice treated with Mel B, suramin and neem extract at 1000mg/kg bwt had significantly greater weights and lived longer than those treated with either water only or lower neem extract concentrations.

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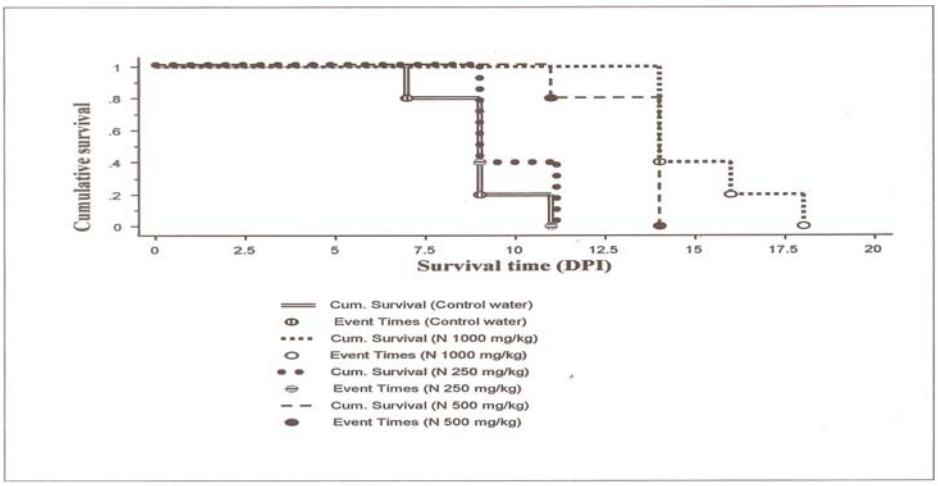

Fig. 6. *T. b. rhodesiense* infected mice treated with neem extracts *ad libitum* at 250, 500 or 1000mg/kg bwt, or water only 24 hours post-infection showed varied survival periods. The survival rates of the neem extract-treated groups were significantly higher than the water-treated controls ($p<0.05$, log rank test).

effect in mammals where no apparent change in relative organ weight, hematological parameters, enzyme levels and histopathology of several organs was recorded in mice with as much as 2g/kg bwt neem extract (21). Indeed, the body weight and PCV levels of all the uninfected animals increased gradually during the period of toxicity testing. These changes correlated very well with data obtained from animals given water only. Except for the Mel-B treated mice, infection with trypanosomes caused parasitaemia and deterioration of health as indicated by a decline in PCV, weight loss and death. There were significant differences in the health parameters in the various neem extract-treated infected mice.

The ability to control parasitaemia level, PCV and also extend survival period of infected mice was dose-dependent indicating that neem extract contains anti-trypanosomal compounds. Of interest was the fact that the 1000mg/kg bwt neem extract was able to delay the appearance of parasites in the blood following infection, control parasitaemia levels, weight loss and also extend the lifespan of the mice with comparable ability to suramin, a known trypanocidal drug.

This anti-trypanosomal effect could be attributed to active neem compounds including azadirachtin, condensed tannins, gallic acid, gallocatechin, epicatechin, catechin and epigallocatechin, three tricyclic diterpenoids: margolone, margolonone and isomargolonone, and polysaccharides isolated from neem stem bark (21). Polyphenols have been reported to have the ability to complex with extra-cellular and soluble proteins and also parasite cell wall thereby disrupting the parasite cell membrane. The site and number of hydroxyl groups on polyphenols are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (25-27). This explains why polyphenols present in neem were more effective in parasite reduction. The ability of the neem plant extract to lower the levels of parasitaemia can therefore be attributed to the toxic activity of polyphenols present in it. Indeed polyphenols have been shown to have anti-trypanosomal activity (26, 28).

Trypanosomosis is a disease whose pathological effects are initiated through the release of cytokines (29) and nitric oxide (30). The improved survival of trypanosome infected mice could be due to the effects of polyphenols in neem extract. Indeed polyphenols in tea have been shown to attenuate cytokine induced (27, 31) and also nitric oxide inflammation (32). At the same time tea protects against nitric oxide toxicity due to the catechins (33).

PCV and anemia are common and critical features in the pathogenesis of African trypanosomosis contributing to morbidity and mortality and thus curtailing the absence longetivity (34, 35). Infected mice given neem extract in this study showed significantly higher levels in PCV compared to the infected mice given water only which can be ascribed to an

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enhanced resistance of erythrocyte haemolysis. This demonstrates clearly that neem extract containing polyphenols like flavoinods posses' *in vivo* ability to protect erythrocytes from haemolysis which can be attributed to flavanoids. In addition, erythrocytes have membranes with a high content of polyunsaturated lipids and a rich oxygen supply making them vulnerable to lipid peroxidation (36). Reactive oxygen radicals generated during infections such as trypanosomosis can attack erythrocyte membrane, induce its oxidation and thus trigger haemolysis (37). However the antioxidant activity of polyphenols (38) might have elicited antioxidant capacity leading to a reduction in the susceptibility of erythrocytes membrane destruction. Indeed treatment of *Trypanosoma brucei brucei* infected rat with vitamins with antioxidant ability ameliorated anemia and organ damage (39, 40).

The observed improved survival of neem treated mice in this study could be related to effects of the active compounds present in neem on red blood cell and/or antioxidant activity. We hypothesize that the ability of neem to prolong life of mice in our study could be attributed to their ability to aid total antioxidant defense system. This has been shown to reduce oxidative stress by protecting the defense system against the damaging effects of reactive oxygen species such as singlet oxygen, peroxyl radicals, nitric oxide and peroxynitrite. Indeed, nitric oxide has been implicated in the pathogenesis of trypanosomosis (30). We can therefore speculate that without the host immunological assistance, high concentration of neem would be necessary to reduce *T. b. rhodesiense* in the host. This indicates the need for detailed mechanistic studies together with the development of parasite-specific drug formulations. This is due to the fact that the current treatment regimens, based on chemotherapy for these parasites are limited and not ideal since they are associated with severe side effects and development of drug resistance (11).

The inability of neem extracts to fully destroy the parasite as is the case with Mel-B could be related to the relatively low amount of the active compounds in the crude neem extract and also the virulence of the parasite species. Indeed, the trypanocidal potential of *A. indica* against *Trypanosoma brucei* has been successfully assessed (16). *Trypanosoma b. rhodesiense* is known to be highly virulent and causes an acute infection. In the current study suramin, a known anti-trypanosomal agent was also not able to eliminate the parasite. The anti-trypanosomal effect of the neem extract can be improved by using a higher dose of the extract or extracting the active ingredient for use in pure form. It is envisaged that the results of the current study will lead to the production of a safe and effective drug for the treatment of human African trypanosomosis. This strategy would be more cost-effective than the tedious and expensive chemical synthesis of trypanocidal drugs, most of which are very toxic.

Acknowledgements

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