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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

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ABSTRACT

Objective: To investigate the effects of combination therapy of methanolic leaf extract of *Azadirachta indica (A. indica)* and diminazene diaceturate (DDA) in the treatment of experimental *Trypanosoma brucei brucei (T. brucei brucei)* infection in rats. **Methods:** Acute toxicity study of the drug and extract combinations were done. Selection of the best drug and extract combinations was carried out using fifty four rats of both sexes separated into 9 groups. Three dose combinations were derived from selection of the best drug and extract combinations used for the final study viz: 7 mg/kg bw DDA plus 125 mg/kg bw extract (group B), 3.5 mg/kg bw DDA plus 250 mg/kg bw extract (group C), and 1.8 mg/kg bw DDA plus 500 mg/kg bw extract (group D). The final study had in addition to the three groups derived from the dose response study, four other groups viz: uninfected untreated negative control (group F), infected and treated with 3 000 mg/kg bw extract alone (group E), infected and treated with 7 mg/kg bw DDA alone (group A), and infected untreated positive control (group G). The parameters assessed were onset of parasitaemia (OP), level of parasitaemia (LOP), clearance of parasites post treatment (COPPT), relapse infection period (RIP), post infection survival period (PIST). **Results:** There was no significant difference in OP between the groups ($P<0.05$). One day post treatment, the mean LOP of groups A, B, and C were found to be significantly lower than that of group D which in turn was lower than that of group E and G respectively. The mean LOP of group E was significantly lower than group G two days post treatment and this trend continued throughout the experimental period. Mean COPPT of group D was significantly longer than that of groups A, C and B. There was no significant difference in the mean COPPT among groups B, C and A. The mean RIP of group D was significantly shorter than group C, and that of group C was significantly shorter than that of group A. There was no relapse of infection in group B. The PIST of group E did not differ significantly from group G. **Conclusions:** This experiment stands to conclude that combination of 125 mg/kg bw extract and 7 mg/kg bw DDA is very effective in the treatment of trypanosomosis, caused by *T. brucei*. This combination therapy proved to be better than single therapy of DDA.

1. Introduction

Trypanosomosis, an infection caused by organisms of the genus *Trypanosoma* is the most economically important disease in sub-Saharan Africa[1]. The loss in livestock production and mixed agriculture alone is valued at 5 billion US dollars yearly in Africa[2]. It is currently estimated that about 60 million people and 48 million cattle[3] are at risk of contracting African trypanosomiasis from the 23 species and 33 sub species of tsetse flies infesting 10 million km^2 of Africa stretching across 40 countries. Tsetse transmitted African trypanosomosis is responsible for 55 000 human and 3 million livestock deaths annually[3] and this hinders mixed farming through reduced work efficiency of draft animals. One most commonly used drug to control and treat the disease is diminazene. Several limitations to the use of diminazene as a trypanocides are high cost[1], drug resistance[4], and relapse of infection[5]. *Azadirachta indica (A. indica)* (Neem) is a medicinal plant used in traditional medicine for the treatment of pests[6], fungal[7], bacterial[8],

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viral[7] and trypanosomal[9,10] infections. In Nigeria, it is popularly called "Dogonyaro". Review of literature revealed that this medicinal plant remains unexplored in the area of combination therapy. With the increasing incidence of drug resistance and non-development or unavailability of new trypanocides, most current research efforts are directed towards making optimal use of the older drugs by combining them with enhancing agents[1,4]. This study was therefore designed to investigate the effects of combination of methanolic leaf extract of *A. indica* and diminazene diaceturate in the treatment of experimental *Trypanosoma brucei (T. brucei)* infection in rats.

2. Materials and methods

2.1. Experimental infection

Each rat was experimentally infected with 1.25×10^5 trypanosomes in phosphate buffered saline (PBS) intra-peritoneally (i.p). The number of parasites was assessed using standard method[11].

2.2. Trypanosomes

Trypanosoma brucei brucei (T. brucei brucei) (field strain) was used for the study. The trypanosomes were obtained from donor rats infected with *T. brucei brucei* isolated from a dog clinically infected with *T. brucei brucei*.

2.3. Diminazene diaceturate

A sachet of 2.36 g Diminaveto®, a product of VMD Holland marketed by TOSAM Nig. Ltd, containing 1.05 g diminazene diaceturate and 1.31 g antipyrine, NAFDAC Reg. No. 04-2816, Man Date = 05-2008, Exp Date = 06-2013, Batch = DG/20278 was properly reconstituted with normal saline and used for the study.

2.4. Experimental animals

Adult albino rats weighing between 100 g and 120 g obtained from the Laboratory animal unit of Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka was used for the study. The rats were housed in clean cages in fly-proof house at room temperature (28-33 ℃) and fed commercial feed (Vital feeds, GCOM Nig. Ltd) and provided with clean water *ad libitum*. The rats were screened for the presence of blood parasites using wet and Giemsa-stained thin films prior to commencement of the experiment.

2.5. Plant material and its extraction

Fresh leaves of *A. indica* were collected from the Nsukka campus of University of Nigeria, Nsukka in June, 2008 and were identified by plant taxonomist in the Botany Department of the University. Detailed description of the plant is available in literature[11]. The fresh leaves of *A. indica* were dried under mild sunlight. The dried leaves were pulverized using mortar and pestle into coarse particles. The material was finely ground with a grinding machine. Cold extraction was performed. 550 g of the powdered plant leaf was extracted in 1 500 mL of 80% methanol with intermittent shaking at intervals of 2 h for 48 h. The extract was filtered using What-man size-1 filter paper and concentrated in vacuo using vacuum rotatory evaporator connected to cold water circulator and pressure pump at 40 ℃ and 210 mmHg pressures. The percentage yield was determined using the formulae: Yield = weight of material obtained divided by weight of starting material multiply by 100 over one. The extract, MAILE was then kept in the refrigerator at 4 ℃ for use in the experiment.

2.6. Experimental design

2.6.1. Experiment 1: acute toxicity

Thirty rats of both sexes divided into five groups of six each were used for the study. The MAILE was given orally and DDA, intra-peritoneally. The combination of DDA and the MAILE administered to different groups of rats is shown below.

Group A: 7.0 mg/kg DDA plus 125 mg/kg MAILE. Group B: 3.5 mg/kg DDA plus 250 mg/kg MAILE. Group C: 1.8 mg/kg DDA plus 500 mg/kg MAILE. Group D: 0.9 mg/kg DDA plus 1 000 mg/kg MAILE. Group E: 7.0 mg/kg DDA plus 3 000 mg/kg MAILE

2.6.2. Experiments 2: selection of the best drug and extract combinations for use in the study

Fifty four rats of both sexes were separated into 9 groups of six rats each were used. All the rats were infected with 1.25×10^5 trypanosomes given intra-peritoneally. DDA was given intra-peritoneally as a single dose, while the MAILE was given orally with the help of intra-gastric tube as a single dose. The treatments were administered when parasitaemia was established in all the rats (six days post infection). The DDA and MAILE combination administered is shown below.

Group 1: 7.0 mg/kg DDA plus 125 mg/kg MAILE. Group 2: 7.0 mg/kg DDA plus 250 mg/kg MAILE. Group 3: 7.0 mg/kg DDA plus 500 mg/kg MAILE. Group 4: 3.5 mg/kg DDA plus 125 mg/kg MAILE. Group 5: 3.5 mg/kg DDA plus 250 mg/kg MAILE. Group 6: 3.5 mg/kg DDA plus 500 mg/kg MAILE. Group 7: 1.8 mg/kg DDA plus 125 mg/kg MAILE. Group 8: 1.8 mg/kg DDA plus 250 mg/kg MAILE. Group 9: 1.8 mg/kg DDA plus 250 mg/kg MAILE

Criteria for selection of best combination: Best combination that cleared the parasites with least dose was given preferential consideration. Any combination that did not clear the parasites after 7 days was discarded and the lowest mean was given preferential consideration. The experiment was terminated 7 days post treatment.

2.6.3. Experiment 3: efficacy study using combinations of DDA and MAILE

Eighty four rats of both sexes divided into seven groups of twelve rats each were used for the study. Six groups were infected with *T. brucei brucei* organisms. Each rat

was infected with 1.25×10^5 trypanosomes given intra-peritoneally. Following the development of parasitaemia (6 days post infection); each group was treated as indicated below.

Group A: Infected and treated with 7.0 mg/kg DDA bw only. Group B: Infected and treated with 7.0 mg/kg DDA bw and 125 mg/kg bw MAILE. Group C: Infected and treated with 3.5 mg/kg DDA bw and 250 mg/kg bw MAILE. Group D: Infected and treated with 1.8 mg/kg DDA bw and 500 mg/kg bw MAILE. Group E: Infected and treated with 3 000 mg/kg bw MAILE only. Group F: Uninfected untreated. Group G: Uninfected untreated.

Efficacy of the combination was determined using onset and level of parasitaemia (OP and LOP), clearance of parasites post treatment (COPPT), relapse infection period (RIP), and death. Moribund rats were euthanized using mild ether anaesthesia and considered as dead animals.

2.7. Parasitaemia

Blood for detection of onset of parasitaemia (OP), level of parasitaemia (LOP), clearance of parasites post treatment (COPPT) and relapse of infection (ROI) was collected from the tail vain of rat by making a nip at the tail end. Blood films were made by placing a small drop of blood from the tail of rat on to a clean glass slide and dropping a clean cover slip upon it and a thumb pressed down upon it carefully so that the blood film spread evenly. The film was carefully examined under the 40× objective of a light microscope for trypanosomes and the level of parasitaemia were assessed using the rapid matching method[9,10]. This was done on daily basis after infection for OP and LOP; after treatment for COPPT and after clearance for ROI.

2.8. Post-infection survival period (PIST)

The rats were observed throughout the period of the experiment. Rats that were moribund were anaesthetised using mild ether anaesthesia and considered dead. The mortality date for each rat in each group was recorded after which the mean post infection survival time of all the groups was calculated.

2.9. Data presentation and statistical analysis

Data obtained were presented in graph for LOP and as means in tables for ROI and COPPT. Data obtained were analyzed using ANOVA followed by Duncan post hoc test and least significant difference (LSD) to determine the significant differences among the means of the various groups. PIST was analysed using student *t*-test. Significance was accepted at the level of $P<0.05$.

3. Results

3.1. Yield of the extract of plant material

The yield of the extract of the plant material was 7.62% of the starting material.

3.2. Acute toxicity

There was no mortality in all the groups at the end of the 3-day study period observed for acute toxicity. The faecal droppings of all the groups were formed and dry except for group E that was pasty. The skin, fur of all the groups looked normal.

3.3. Results of selection of the best DDA and MAILE combinations for use in the study

The parasites in all the rats in groups 1, 2, 3, 5 and 6 were cleared two days post treatment. Parasites in rats in group 4 were cleared three days post treatment. The parasites in all the rats in groups 7 and 8 were not cleared seven days post treatment. However, the parasites in group 9 were cleared three days post treatment. The dose used for group 1, 5, and 9 were chosen for further study.

3.4. Results of efficacy study using combinations of DDA and MAILE

3.4.1. Onset of parasitaemia

Trypanosomes were first noticed in the blood of infected rats in groups C and D four days post infection and by the sixth day post infection, trypanosomes were detected in all the infected rats. Though trypanosomes appeared first in groups C and D, the onset of parasitaemia in the infected groups were not found to differ significantly ($P<0.05$).

3.4.2. Level of parasitaemia (LOP)

The results of the level of parasitaemia (LOP) revealed that six days post infection, the mean LOP was not found to differ significantly in all the infected groups. However one day post treatment, the LOP of groups A, B, and C were found to be significantly lower than that of group D which in turn was lower ($P<0.05$) than that of group E and G respectively. And that of group E was significantly lower than that of group G three days post treatment and this trend continued throughout the experimental period (Figure 1).

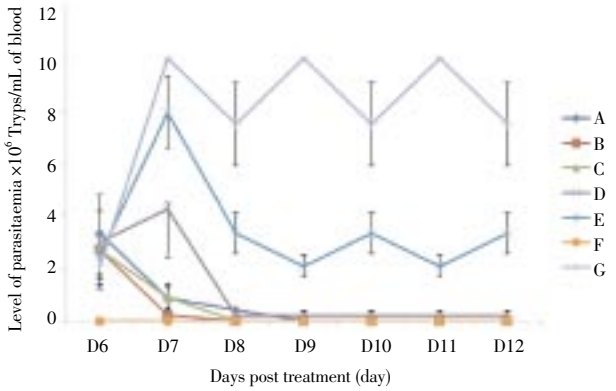

Figure 1. The level of parasitaemia in rats infected with *T. brucei brucei* and treated with different combinations of DDA and MAILE. Note: Treatment was day 6 post infection.

3.4.3. Clearance of parasites post treatment (COPPT)

There was 100% clearance of parasites in groups A, B and C whereas 83% of group D parasites were cleared and groups E and G had 0% parasite clearance respectively. The mean COPPT of rats in group D was significantly longer than that of groups C and B. There was no significant difference ($P<0.05$) in the mean COPPT among groups B, C and A; and between groups A and D.

3.4.4. Relapse of infection period (RIP) after clearance

The mean RIP of group D was significantly shorter than that of group C, and that of group C was significantly shorter ($P<0.05$) than that of group A. There was no relapse of infection in group B.

3.4.5. Post infection survival time (PIST)

Rats in groups A, B, and C did not die throughout the experimental period. Group D had 17% mortality and groups E and G had 100% mortality respectively. When the mean PIST of group E was compared to group G; it was not found to differ ($P<0.05$) significantly.

4. Discussion

The results revealed that of all the doses used for acute toxicity, none of the rats in any of the groups died. According to WHO acute hazard rankings, the extract can be described as unlikely to present hazard in normal use since the maximum dose of 3 000 mg/kg body weight (bw) did not lead to mortality[13]. In addition, the fact that at the highest dose combination (7 mg/kg bw DDA and 3 000 mg/kg bw extract); none of the rats died indicated that the combination is unlikely to present acute hazard in normal use. The parasites in all the rats in groups 1, 2, 3, 5 and 6 were cleared two days post treatment. This indicated therapeutic superiority of dose combinations used in those groups probably due to synergy and potentiation. Parasites in rats in group 4 were cleared three days post treatment. The parasites in all the rats in groups 7 and 8 were not cleared seven days post treatment. However, the parasites in group 9 were cleared three days post treatment. This result indicated that the higher the dose of diminazene and lower dose of extract the better the therapeutic effect. Higher dose of the extract and lower dose of diminazene is also of therapeutic effect provided the diminazene is not less than 1.8 mg/kg bw. The dose used for group 1, 5, and 9 were chosen for further study. The results of efficacy study using combinations of DDA and MAILE, showed no significant different in the mean OP in all the infected groups six days post infection, similar in effect to the dose response experiments. However, the mean LOP differed shortly after treatment among the groups. Group B (treated with 7 mg/kg bw DDA plus 125 mg/kg bw MAILE) cleared the parasites fastest and group C (treated with 3.5 mg/kg bw DDA plus 250 mg/kg bw MAILE) cleared the parasites faster than group A (treated with 7 mg/kg bw DDA alone) in absolute values may be attributed to the fact that the extract have synergistic effect with diminazene. The mean COPPT of group D was significantly longer than that of groups A, C and B. There was no significant difference in the mean COPPT among groups B, C and A. This therapeutic superiority which group B has over C, and C over A, regarding absolute values of mean COPPT, was equally exhibited significantly by groups B, C and A over group D. Group E had significantly lower mean LOP when compared to group G and this reduction continued throughout the experimental period suggesting strongly that the extract on its own had trypanocidal effect, a finding consistent with the findings of other researcher[8]. In addition, the fact that group B had 0% relapse infection, group A, 20% relapse infection and group C 40% relapse infection and group D, 100% relapsed infection showed pharmacological superiority of the dose combination used in group B over doses used in group C, A and D respectively. Again, group B had no relapse of infection whereas the mean RIP of group D was significantly shorter than that of group C, and that of group C was significantly shorter than that of group A, showing pharmacological superiority of the dose combination used in group B over doses used in group C, A and D respectively. This pharmacological superiority may be attributed to at least three factors: (1) The extract have trypanocidal effect on its own, a finding observed by significantly reduced level of parasitaemia in group E compared to group G two days post treatment, a trend that continued throughout the experimental period. (2) The extract may have assisted the diminazene to access sites that ordinarily are difficult for diminazene to penetrate and destroy all the parasites at those sites as observed in lack of relapsed infection in group B when compared to group A. (3) It may also be that the extract prolonged the half-life of the diminazene, such that the drugs stayed longer in the blood than diminazene alone and exert longer trypanocidal activity than the single therapy observed as zero percent relapsed infection in group B and 20% relapsed infection in group A. It should be noted that the genetic structure of trypanosome population (clonal or panmictic) is an important parameter influenced by the transmission intensity, and this in turn might influence the rate of development of drug resistance. This has been suggested by studies on drug resistance in plasmodium, which has shown that parasite populations in infected hosts are polyclonal with differing drug sensitivities between the clones making up the population. It is likely that a similar situation occurs in trypanosome infections[1]. This difference in drug sensitivity is probably why the DDA and the extract combination were able to clear the trypanosomes which DDA alone could not eliminate and without relapse as seen in group B. The relapse of infection seen in group A may be attributed to the fact that the population sensitive to DDA were cleared while those not sensitive to it remained

but because of the diagnostic technique used and the relative little quantity of the parasites left in the blood, there appeared to be total clearance of parasites. However, with drug biotransformation and elimination as well as with the multiplication of those parasites not sensitive to DDA, the parasite multiply and attain a detectable level and was observed as relapse of infection seen in group A when compared to group B. This population not sensitive to DDA could be attributed to be resistant to DDA [3]. The observed relapse therefore could be attributed to resistance and reappearance of parasites from site not accessible to trypanocidal agents (relapse). The aforementioned reasons as well as sub therapeutic dosage and probably immunosuppression and cross resistance can be attributed to the relapse observed in group D, C and A respectively [1].

This relapsed infection was observed by other workers[5] at days 42 and 49 post treatments with diminazene aceturate after the rats were infected with *T. brucei* alone; and *T. brucei* and *T. congolense* combination respectively. Group C, though had faster clearance rate in absolute values when compared to group A, had significantly reduced mean RIP probably because of reduction in half-life of dose used in group C compared to group A.

The fact that there was 83% clearance in group D and later 100% relapse infection in a much shorter period after clearance may be attributed to the fact that the dose was not therapeutic enough to clear parasites. The 83% clearance was probably due to synergy and potentiation of DDA by the MAILE combination, and the 100% relapsed infection may be due to reduction in half-life (shorter duration of action) occasioned by rapid biotransformation and excretion in that group compared to groups A, B and C. This reduction in half-life phenomenon needs further investigation. Rats in groups A, B and C did not die throughout the experimental period probably because of better therapeutic efficacy of the doses used. Group D had 17% mortality showing that the dose combination used was not as efficacious as the doses used in group A, B and C respectively for the treatment of *T.brucei brucei* infection. The 100% relapse infection in the ones cleared by the dose combination used in group D suggested that this combination is not therapeutic in the treatment of trypanosomosis caused by *T. brucei brucei*.

The mean PIST of group E when compared to group G did not differ significantly which indicated that the extract alone did not have any significant effect on survivability. However, rats in group E have significantly ($P<0.05$) lower LOP than that of group G throughout the experimental period. This is an indication that the extract may have trypanocidal effect, but the effect may not be significant enough for possible therapeutic application alone. This therapeutic possibility may be enhanced if treatment is repeated. Isolation and concentration of active ingredient may equally enhance therapeutic efficacy. In conclusion, this study has indeed established that the combination of 125 mg/kg bw extract plus 7 mg/kg bw DDA is very effective in the treatment of trypanosomosis caused by *T. brucei brucei*, as it quickly cleared the parasites and prevented relapse infection. It can be suggested from the deductions of the study that *A. indica* enhanced diminazene in producing its trypanocidal activity in at least three possible different ways namely: potentiation, synergy and increase in half-life.

Conflict of interest statement

We declare that we have no conflict of interest.

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