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Evaluation of the efficacy of combination therapy in *T. b .brucei* – infected mice using extracts of *annona senegalensis* and *eucalyptus camaldulensis*

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Abstract—African trypanosomiasis is a parasitic disease affecting both humans and animals. Over 60 million people and 50 – 70 million animals are reported to be exposed to the disease. One of the major problems besetting the chemotherapy of the disease is parasite resistance to the available drugs. An attempt was made in this study to explore the potentials of combination therapy using methanol extracts of Annona senegalensis (leaf) and Eucalyptus camaldulensis (leaf) in different combinations to treat T. b. brucei - infected mice. One animal in the group treated with a combination of crude methanol extracts of A. senegalensis and E. camaldulensis (1:1) had parasites cleared from circulation two weeks into the treatment period and continued to survive for more than three months. The untreated control died 2 weeks post-infection while the other groups did not survive beyond 20 days. The weight of the surviving animal increased tremendously over the period and infectivity tests using blood and cerebrospinal fluid from the animal were negative because parasites never appeared in the circulation of sub- inoculated animals. However, histopathological studies of the kidney and liver of the survived animal showed widespread intra renal tubular necrosis with thrombi formation and oedema of the hepatocytes. We conclude that in spite of the apparent long-term effects on the kidney and liver, the methanol extracts of the two plants, acting synergistically, possess appreciable potential for the development of drug combinations to overcome the problem of parasite resistance to conventional drugs.
Keywords—Annona senegalensis; Chemotherapy; Combination therapy; Eucalyptus camaldulensis; Trypanosomiasis.

I. INTRODUCTION

Sleeping sickness, or Human African Trypanosomiasis (HAT), is a fatal and much neglected disease that plagues parts of Africa. The parasite causing sleeping sickness is transmitted to humans through the bite of the infected tsetse flies breeding in warm and humid areas. Inhabiting the vast savannah across sub-Saharan Africa, tsetse flies come into contact with man, cattle and wild animals, all acting as reservoirs for the parasites.Tsetse flies are found in 36 countries in sub-Saharan Africa, thereby exposing about 60 million people to risk of infection. According to World Health Organization (WHO) figures, trypanosomiasis affects 50,000 to 70,000 people each year ([1]). The disease was nearly eliminated in the 1960s, but has made a comeback of epidemic proportions due to war, population movements, and the collapse of health systems over the past two decades.

The disease affects mostly poor populations living in remote rural areas of Africa. Untreated, it is usually fatal. Travellers also risk becoming infected if they venture through regions where the insect is common. Generally, the disease is not found in urban areas, although some cases have been reported in suburban areas of big cities in some disease - endemic countries ([2]).

The first stage of sleeping sickness presents with non-specific symptoms such as fever and weakness. This stage is difficult to diagnose but relatively easy to treat. If no treatment is given, the parasite will invade the infected person's central nervous system and the second stage sets in. This stage may be characterized by confusion, violent behaviour or convulsions. If left untreated, the disease inevitably leads to coma and death ([3]).

A major problem besetting the chemotherapy of African Trypanosomiasis is parasite resistance to the few available drugs ([4]). Recent research suggests a safe, alternative treatment is available through the combination of two drugs, eflornithine and nifurtimox. While this combination represents an improved therapy for patients, it is not ideal. The treatment is complicated to administer and requires close patient monitoring—

something frequently unavailable in sub-Saharan Africa. Hence, major efforts are needed to bring truly innovative drugs into the pipeline. Drug combinations can potentially avert or delay the emergence of drug-resistant organisms. Dosage reductions of each drug combined may reduce the overall toxicity while maintaining good efficacy. Combinations may also allow for a simpler administration of treatment, improving the feasibility of therapy in remote areas with logistic and staffing limitations. Combinations also help reduce risk of drug resistance development ([5]; [6]). In traditional medical practice, the predominant trend is combination therapy, where a number of plants are combined and boiled together in a pot for the treatment of diseases. *Annona senegalensis* Pers. (Annonaceae), commonly known as wild custard apple and locally called ''Gwandar daji'' in Hausa language, ''Abo'' in Yoruba, ''Uburu ocha'' in Ibo, is widespread in the Savannah region of Nigeria especially near streams. The plant decoction has been reported to be used traditionally in the treatment of sleeping sickness ([7]) and in the treatment of cancer ([8]). *Eucalyptus camaldulensis* (leaf) is boiled in combination with other plant parts for the treatment of malaria and typhoid fever in some parts of Northern Nigeria (Undocumented). Scientific evaluations of the extracts of *A. senegalensis* and *Eucalyptus camaldulensis* have demonstrated *in vivo* trypanocidal activity ([3], [9], [10]).

In this study, an attempt was made to mimic and evaluate the efficacy of combination therapy using extracts of *Annona senegalensis* (leaf) and *Eucalyptus camaldulensis* (leaf) in different ratios in the treatment of *T. b. brucei* - infected mice.

II. MATERIALS AND METHODS

2.1 Plant Materials

Annona Senegalensis (NIPRD/H/5868) was obtained fresh from the bush along Minna – Bida road in Niger state, Nigeria, between the months of May and June.

Eucalyptus Camaldulensis (NIPRD/H/6263) was collected from the Education Resource Center/ETF ground in Minna, Niger State, Nigeria, between the months of June and July. The two plants were identified and assigned specimen voucher numbers at the herbarium section of the National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

2.2 Experimental Animals

Albino mice, used for screening, were purchased from the Biochemistry and Chemotherapy Division of the National Institute for Trypanosomiasis and Onchocerciasis Research, Vom, Plateau State, Nigeria. All experiments involving the animals were conducted in compliance with the internationally accepted principles for laboratory animal use and care as contained in the Canadian Council on Animal Care (CCAC, 1997) guidelines on animal use protocol review ([11]).

2.3 *Trypanosoma brucei brucei*

A stabilate of *trypanosoma brucei brucei* (Lafia strain) was obtained from the National Institute for Trypanosomiasis and Onchocerciasis Research, Vom, Plateau State, Nigeria, and subsequently maintained in the laboratory of the Biochemistry Department, Federal University of Technology, Minna, Niger State, Nigeria, by serial passage in mice.

2.4 Preparation of Plant Samples

The leaves of the two plants were removed fresh, washed with running tap water and dried at room temperature. The dried leaf samples were blended into powdered form using an electric blending machine. The powdered samples were stored in clean polythene bags until required for use.

2.5 Preparation of crude extracts

The extracts were prepared using the method described by ([3]). Fifty grams (50g) each of the dried powdered samples of the leaves of *A. senegalensis* and *E. camaldulensis* were extracted sequentially under reflux using 400mls of methanol for 2hrs in each case. Extracts were filtered hot using cheese cloth and solvent was removed using rotary evaporator. The extracts, now in relatively dry form, were transferred into sterile sample bottles for storage in the refrigerator until required for use.

2.6 Infection of animals

This was done according to the method described by ([3]). Blood from a highly infected mouse was obtained by cardiac puncture and collected with EDTA coated syringe. The blood was appropriately diluted with physiological saline to serve as inoculums. Healthy mice of weight range, 25 - 35 g were infected with 0.1ml of the inoculums containing about 10^3 trypanosomes.

2.7 Administration of combined extracts

Combination of extracts was based on a random method using specific ratios to mimic what is done in traditional medical practice. Five groups, each containing three infected mice, were set up. Groups A, B, C were administered intraperitoneally 200mg/kg bodyweight/day of combinations of crude methanol extracts of *A. senegalensis* (leaf) and *E. camaldulensis* (leaf) in ratios 1:1, 1:2, and 2:1 respectively. Group D was administered 3.5mg/Kg bodyweight of berenil to serve as positive control while Group E was left untreated to serve as negative control. Treatment for the test groups lasted 21 days and parasitemia was monitored for one

month. The percentage parasitemia, initial and terminal weights of animals were determined in the course of treatment.

2.8 Monitoring the course of parasitemia

The course of parasitemia was monitored using a microscope set at X 40 based on the method described by ([9]).

2.9 Monitoring changes in percentage packed cell volume (%PCV) and weight of test animals

The initial and terminal % PCVand weights of mice in all groups were noted before infection and after treatment.

2.10 Blood and Cerebrospinal (CSF) infectivity tests

Both tests were carried out according to the method described by ([3]). The only animal that survived for three months after treatment with a combination of the methanol extracts of *A. senegalensis* and *E. camaldulensis* was used to test for blood and CSF infectivity.

2.11 Blood Infectivity Test

The surviving mouse was sacrificed 12 weeks post-treatment and 0.02ml of diluted blood sample obtained from the punctured heart of the mouse was sub-inoculated into three clean parasite-free mice, and parasitemia was monitored three times a week under the microscope over a 2-month period.

2.12 Cerebrospinal Fluid (CSF) Infectivity Test

To obtain CSF, the hair on the back of the surviving mouse was carefully shaved and under a mild anaesthesia, the backbone was positioned such that the head touched the limbs. This made the vertebrae conspicuous. The lumbar was then punctured by the insertion of a clean needle and syringe, into which a clean, clear, and transparent fluid (CSF) flowed. Two clean, parasite-free mice were then sub-inoculated with 0.02ml of the CSF, and parasitemia was monitored for over a two month period.

2.13 Histopathology

The tissues were prepared according to the method described by ([12).The liver and kidney of the sacrificed mouse was carefully removed, processed, sectioned and stained according to standard laboratory methods. These organs were subjected to microscopic examination to establish any changes in their morphology.

III. RESULTS

3.1 Anti-trypanosomal activity of extracts

The anti – trypanosomal activity criteria was judged on the ability of the extracts to clear blood – stream trypanosomes and prolongs the life span of treated *T. b. brucei* – infected mice when compared to the untreated control.

Only the combination of methanol extracts of *A. senegalensis* (leaf) and *E. camaldulensis* (leaf) (1:1) resulted in the complete clearance of parasites from the circulation of one animal in the group (Fig.1). The animal survived for more than two months and was used for blood and CSF infectivity tests. Other combinations used for treatment of *T. b. brucei* – infected mice did not clear parasites completely from circulation although they all survived beyond the life span of the untreated control.

3.2 Changes in weight and % PCV

After treating for three weeks and the mouse surviving for more than 60 days, the terminal weight and % PCV were compared with the initial values as shown on Fig. 2. The weight increased by about 62.5%, from 25g to 40.45g. The %PCV on the other hand increased appreciably from 40 to 43 %.

3.3 Histopathology

Plate 1 represents the micrograph of liver of a *T. b. brucei*-infected mouse after treating with 200mg/kg bodyweight of combined therapy (methanol extracts of the leaves of *A. senegalensis* and *E. camaldulensis*) for three weeks and surviving for more than 60 days. Oedema of the hepatocytes was observed (as indicated by the arrow head on plate 1).

Plate 2 represents the micrograph of kidney of *T. b. brucei*-infected mouse after treatment with 200mg/kg bodyweight of combined therapy (methanol extracts of the leaves of *A. senegalensis* and *E. camaldulensis*) for three weeks and surviving for more than 60 days. It shows widespread intra renal tubular necrosis with micro thrombi formation (indicated by the arrow head on plate 2).

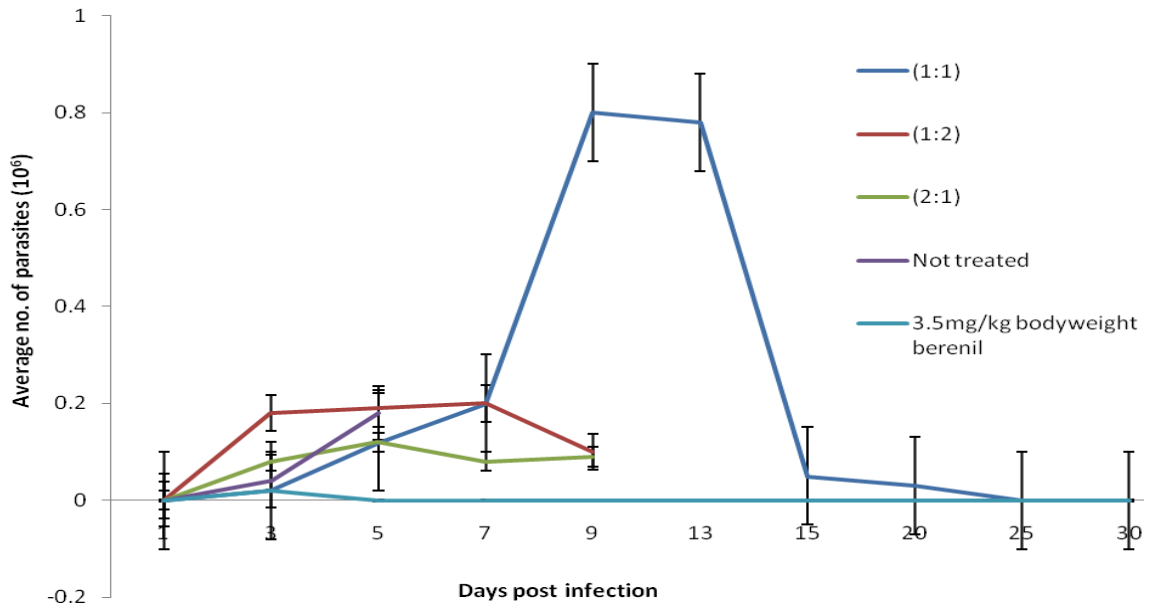

Fig. 1: Course of parasitemia in infected mice treated with combination of methanol extracts of *A. senegalensis* and *E. camaldulensis* (200mg/kg bodyweight) for three weeks at ratios 1:1, 1:2 and 2:1. Complete parasite clearance was obtained in the group treated with 1:1 combination.

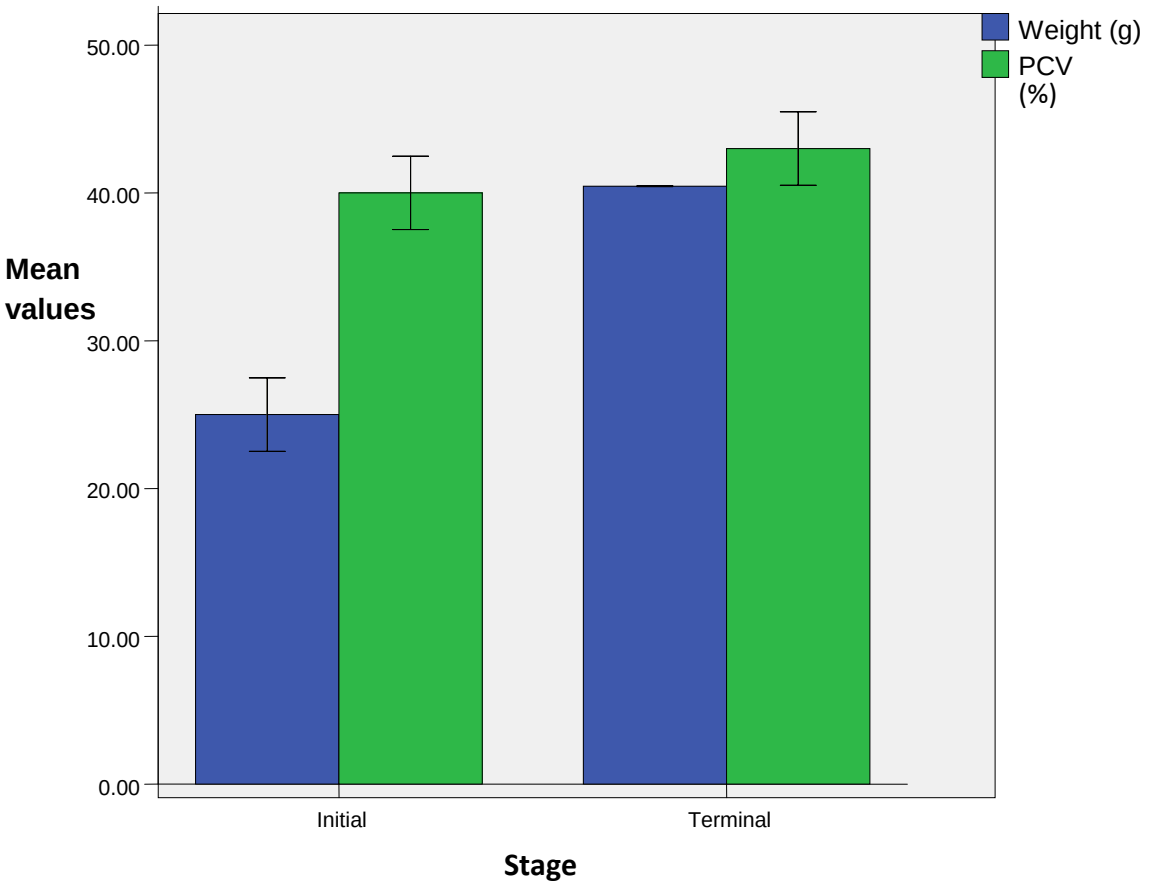

Fig. 2: Initial and Terminal values for weight (g) and % PCV of infected mouse that survived with combination therapy (1:1)

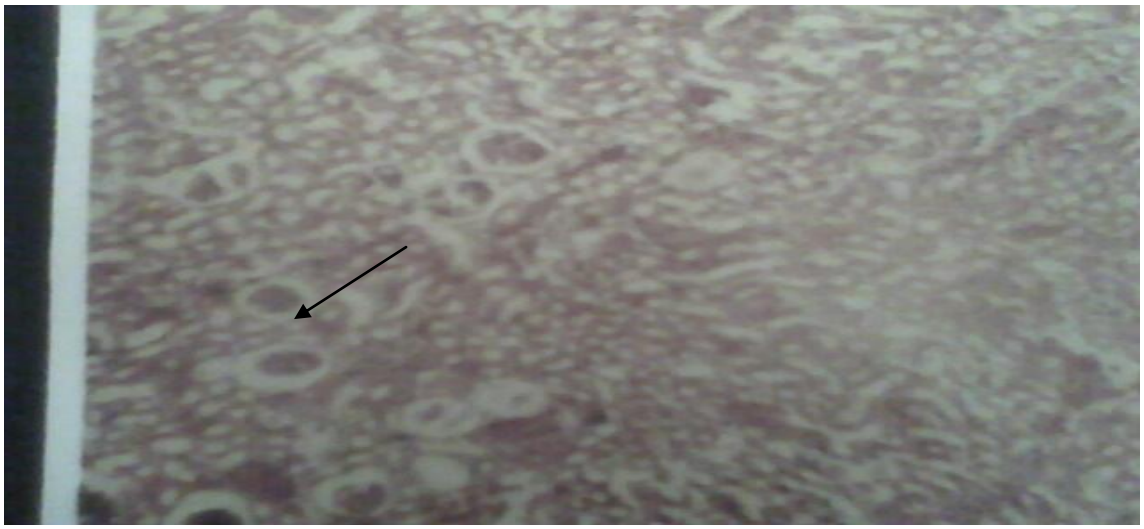

Plate I: Micrograph of liver of a *T. b. brucei* - infected mouse at X 100 treated with 200mg/kg bodyweight of combined therapy (A.S : E.C). There is edema of the hepatocytes (arrow) due to long term effect of the extracts. (3 months later)

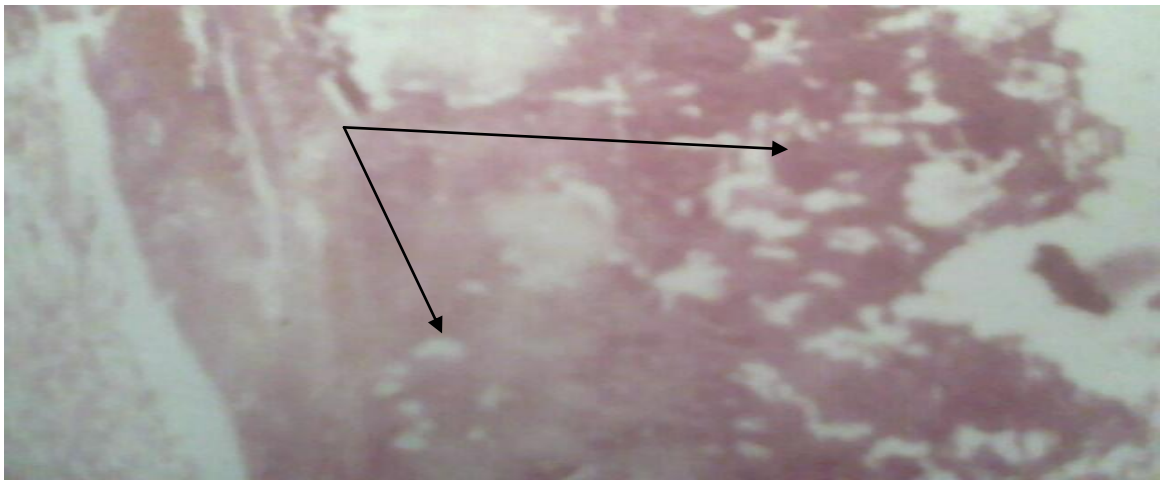

Plate II: Micrograph of kidney of *T. b. brucei* - infected mouse at X 100 treated with combined therapy. There is intra renal tubular necrosis with micro thrombi formation (arrow heads), three (3) months later.

IV. DISCUSSION AND CONCLUSION

Drug combinations can potentially avert or delay the emergence of drug-resistant organisms and dosage reductions of each drug combined may reduce the overall toxicity while maintaining good efficacy ([13]). In this study, only one combination (1:1) of the methanol extracts of *A. senegalensis* and *E. camaldulensis* given at a dose of 200 mg/kg bodyweight demonstrated a significant anti-trypanosomal effect on *T. b. brucei* – infected mice (Fig. 1). Mice in this group had blood – borne parasites cleared from circulation of infected mice after treating for three weeks and continued to survive without any parasite for about four months. Cerebrospinal fluid and blood infectivity tests using the mice that survived longest showed that it was completely free of parasites, thus further confirming the efficacy of the combination in the treatment of animal trypanosomiasis. The efficacy of drug combination as an effective means of countering parasite resistance and that of synergism between artemisinin and a medicinal plant, *Erythrina abyssinica* (Lam. ex) against *Plasmodium falciparum* parasites have been demonstrated ([14], [5], [6], [15]).

It is interesting to note that the result obtained in this work has demonstrated the mechanism of synergy between the two extracts in potentiating the activity of one another. In conventional medical practice, combination therapy has virtually taken precedence over mono - therapy in overcoming resistance by pathogens. It is therefore not surprising that the combined extracts demonstrated an encouraging anti - trypanosomal activity. This result without doubt, is a justification for the traditional practice by herbalists who combine multiple plants to treat a single disease condition, but it has also demonstrated that efficacy depends on appropriate ratios of combinations between the plants being used.

It was also observed in this study that treatment with this combination resulted in a tremendous increase in the weight and an appreciable rise in percentage packed cell volume (%PCV) of the treated animals (Fig. 2). These observations give credence to the ability of the combination to reverse the two principal symptoms of African trypanosomiasis namely, loss of weight and anaemia. Although the histopathological profile of the liver and kidney of the animal showed oedema of the hepatocytes (plate I), and widespread intra renal tubular necrosis with micro thrombi formation in the kidney of the treated animal (plate II), an indication of hepatotoxicity and nephrotoxicity, there is room for improvement and possible perfection of the protocol with a view to preventing these undesirable side effects. It is very likely that when the crude extracts are subjected to fractionation to obtain purer and more active fractions, the side effects observed will be minimised.

Though this study is a preliminary one, the mere fact that some positive indices like clearance of parasites and prolongation of life span were recorded for the treated group is an interesting development because this is the first reported *in vivo* screening for anti - trypanosomal activity using combination of extracts from these two plants or in fact any other combination of plant extracts.

We strongly believe that this combination therapy project can be carried forward bearing in mind the fact that the practice among traditional medicine practitioners in Nigeria is to combine an array of medicinal plants for the treatment of single or multiple diseases and in most cases these combinations have produced positive results. What this screening has succeeded in doing is to establish the possibility of synergistic action by plant extracts in the treatment of diseases using animal models and this will provide a basis for further studies using combinations of extracts from different plants to tackle the ever – increasing phenomenon of parasite resistance to existing pharmaceuticals.

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