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COMPARATIVE ANTITRYPANOSOMAL SCREENING OF METHANOLIC EXTRACTS OF KHAYA SENEGALENSIS AND MORINGA OLEIFERA

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ABSTRACT
The in vitro and in vivo activities of methanolic extracts of defatted leaves and stems of Khaya senegalensis and Moringa oleifera on Trypanosoma brucei brucei were investigated and compared. The in vitro assessment involved incubating the parasite (in triplicate) in the presence of various extract concentrations in a 96-well microtitre plate against negative and drug controls. The stem extract of Khaya senegalensis and Moringa oleifera stem extract gave the highest and the least in vitro activities with per cent drop in parasite population of 43.76 and 29.46 respectively. The in vitro results compared well with that of Diminazine Aceturate, a standard trypanocides employed in the control well. Intraperitoneal treatment of T. brucei brucei-infected mice using these stem extracts at 200 mg/kg b.w. revealed complete elimination of the parasite from blood circulation in Moringa oleifera –treated animals and suppression of parasitaemia in Khaya senegalensis-treated group, contrary to expectation raised by the in vitro findings. Subinoculation experiment with various organ homogenates, however, showed residue of live parasite hidden within organs of infected animal whose parasites were cleared from systemic circulation. The implications of these findings is discussed.

Keywords: Comparative, Antitrypanosomal activities, Khaya senegalensis, Moringa oleifera, Trypanosoma brucei brucei, Traditional medicine.

INTRODUCTION

African trypanosomiasis,commonly known as sleeping sickness, is a vector-borne parasitic disease of man and his domestic animals. Trypanosome, the causative parasites of this infection, is a protozoan belonging to the genus trypanosoma. The parasites are transmitted to their human and animal hostsvia tsetse fly bites which have acquired their inflection from human or from animals harboring the human pathogenic parasite.

Animal trypanosomiasis is a major factor retarding the growth of the livestock industry in Africa and it has constituted a major obstacle to the economic development of the rural areas affected.The disease has undergone a dramatic and devastating resurgence in recent years especially in sub-Saharan Africa (Welburn et al, 2001) and thus an important priority for biomedical and public agencies, agricultural sector and the scientific community (Aksoy, 2003).

Attempt to develop trypanosomal vaccine has been frustrated by antigenic variation, and chemotherapy, which is currently the main management strategy being adopted against the disease, is faced by numerous challenges such as drug toxicity, drug resistance and high cost of producing synthetic compounds especially for developing countries (Gutteridge, 1985; Kuzoe, 1993). Therefore there is the need to search for the cheaper, more effective, easily available and less toxic chemotherapeutic agents for combating the disease. The use of herbal preparations in traditional medicine has encouraged the sourcing of antidisease agents from indigenous vegetation (Nok et al 1993 and Atawodi, 2005).

Khaya senegalensis (juss), a dry zone mahogany belonging to the family meliaccare, and Moringa oleifera commonly called Drumstick, horseradish tree, radish tree or Ben nut tree (belonging to the family, mirongaceae (Aliyu, 2007), are popular medicinal plants of northern Nigeria (Asuzu and Cheneme, 1990; Ibrahim et al., 2008). Survey work (Atawodi et al, 2002) has suggested their possible role in the treatment of infection due trypanosomes. Scientific efforts towardsscreeningthese plants for their antitrypanosomalare scanty (Atawodi et al., 2003; Wurochekke and Nok, 2005). This paper presents a comparative study on the antitrypanosomal activities of these plants at in vitro and in vivo level.

MATERIALS AND METHODS

Test organism

The test organism used, Trypanosomabruceibrucei, was obtained from stabilates maintained at the Nigerian Institute for Trypanosomiasis Research (NITR), Kaduna, Kaduna State, Nigeria. The parasite was maintained in the laboratory by serial passage in rats. Passage into healthy rats was considered necessary when the parasitaemia was in the range of 100 and above per microscopic field (at X400 magnification).

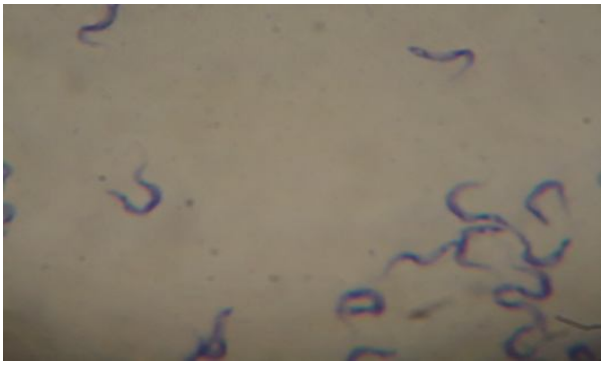

Fig. 1. Giemsa stained picture of T. brucei brucei (X4000 magnification)

Experimental animals
White Albino rats and Mice of both sexes were raised in the animal house of the Department of Biochemistry, Kaduna state University, Kaduna Nigeria. All the animals were kept in a well ventilated laboratory cages, were maintained on a commercial poultry feed (Fitzer, Nigeria) and given water al libitum. The mice weighted between 18-25g while the rats weighed between 120-150g.

Plant collection
The stem and leaves of mature Khayasenegalensis and Moringaoleifera were collected from Zaria metropolis in Zaria local government area of Kaduna state, Nigeria. The plants were thoroughly washed and oven dried at 40 0C to a constant weight then pounded to a fine powder using a ceramic pestle and mortar. The powder samples were then extracted as described below.

Plant extraction
Exactly 25 g of each plant sample was oven dried at 40oc and weighted using an electronic weighing balance, then socked in 150 ml petroleum ether (40 oC – 60 oC) and allowed to extract for two days with frequent intermittent shaking. The extract was then filtered through muslin cloth followed by filtration through filter paper. The residue was refluxed in 150 ml methanol for 2-day with shaking as above. Both filtrates obtained from petroleum ether and methanol extraction were evaporated using rotatory evaporator followed by evaporation to dryness in a water bath at 40 0C. The residues were collected, their weights noted and stored in a refrigerator at 4 0C until required in sample bottles.

Culture Medium
The medium used for the in vitro culturing of Trypanosoma brucei brucei contained amino acids, antibiotics, vitamins and inorganic salts as reported by Eagle (1955) which was manually constituteds. The medium was supplemented with 1% (w/v) D-glucose and 20% heat inactivated horse serum and its pH adjusted to 7.4.

Maintenance and harvesting of trypanosomes
The parasite was grown in healthy laboratory white albino rats and was harvested from infected rats with high parasitaemia. At peak parasitaemia, rats were anaesthetized with chloroform, sacrificed and their blood collected using syringe containing phosphate buffer saline with 1% (w/v) EDTA via cardiac puncture. The blood was centrifuged at 3000 rpm for five minutes. The parasites were collected from the Buffy coat region and transferedinto phosphate buffer saline glucose (PBSG), pH 7.4.

Determination of Parasitaemia
Parasitaemiain infected rats was monitored using the method of Herbert and Lumsden (1976). Briefly, ethanol-sterilized animal's tail was cut open with a blade and a drop of blood placed on a clean glass slideandimmediately covered with a clean cover slip. The wet smear was then viewed microscopically at x400 magnification. The number of parasites counted per microscopic field was compared and matched with standard values provided by Herbert and Lumsden. Corresponding logarithmic values were converted to antilogarithm in order to obtain absolute number of trypanosome per ml of blood.

In vitro study with methanolic crude extracts of Khaya senegalensis and Moringa oleifera
Exactly 60 mg of each extract was weighted and dissolved in 3ml of culture medium, serial dilution of this stock solution was done using the culture medium to obtain various concentrations (3000µg/ml,1000 µg/ml, and 300µg/ml). Assessment of the in vitro antitrypanosomal activity was performed in triplicates in 96 wellsmicrotitre plates (flow laboratories Inc., Mclean, Virginia, USA). Exactly 50µl of each extract was incubated in microtitre wells at 37 0C with 20 µl of the parasite suspension (density of about 12 106 parasite/ ml). The control well contained 250 µl ofculture medium with parasites except that no extract solution was added to it. The drug control wells, on the other hand,contained 13 µg/ml of diminazene aceturate in the culture medium. Parasite motility was monitored on a glass slide covered with a covering slip under a microscope at ×400 magnification. The number of motile parasites was counted before and two hours after the addition of extracts/drug. Drop in the percentage of the motile parasites was taken as an indication of extracts activity against Trypanosoma brucei brucei for the period of two hours employed.

Study on the in vivo antitrypanosomalactivity of methanolic extracts of Khaya senegalensis and Moringa oleifera.
Exactly nine (9) mice were divided into 3 groups of 3 mice each. Each mouse in all groups was intraperitonially inflected with 1.2x106 Trypanosomes. The level of parasitaemia was monitored daily using the method of Herbert and Lumsden (1976) throughout the duration of the experiment. After infection was established (3 days post infection) each mouse in the first two groups was intraperitonially treated with 200mg/kg b.w.of stem extracts of Khaya senegalensis and Moringa oleifera respectively. The third group was left as a positive control (with not treatment). The levels of parasite in mice blood were monitored daily thereafter until death or end of the experiment. Values obtained were compared.

Subinoculation Experiment
A treated mouse in Moringa oleifera-treatment group was found to have no parasite in the blood for more than a week. This animal was sacrificed two weeks post treatment and its brain, liver, kidney and spleen were taken, rinsed with PBSG and homogenized with the same buffer. The homogenate of each organ was stored in separate container with about 3ml cold PBSG, pH 7.4. About 0.2 ml of each homogenate was used to inoculated three (3) healthy mice (per organ). Their daily parasitaemia were monitored microscopically for possible infection as previously described.

RESULTS

From Table 1, the results for in vitro study on the antitrypanosomal potentials of the extracts revealed highest activity in the methanolic stem extract of Khaya senegalensis having percentage mortality of 43.76 % at lowest concentration of 300 µg/ml while the least activity was found in Moringa oleifera stem extract (29.46 % Mortality). The standard drug, dimnazine aceturate gave an acticity of 36.06 % at a concentration of 1 µg/ml.

The in vivo study (Fig. 2) on the other hand revealed suppression of parasitaemia in the mice group treated with methanoloic extract of Khaya senegalensis stem and showed a near-total elimination of blood parasite by Moringa oleifera extract. In the same way, the animal group treated with Moringa oleifera extract survived longer after treatment, than those treated with methanoloic extract of Khaya senegalensis stem (Fig. 3).

Subinoculation experiments were conducted by separately inoculating groups of healthy mice with homogenates (of Liver, Spleen, Kidney and Brain) obtaned from treated mice from whose blood no circulating trypanosomes were seen. Table 2 shows that all inoculated mice develop infection and attained peak parasitaemia ten days post inoculation.

Table 1. In vitro effect of Moringa oleifera and Khaya senegalensis methanolic extracts on % mortality of T. brucei brucei

Plant	Plant Parts	Drop in parasite population (%)		
		Concentration of extract (µg/ml)		
		3000	1000	300
Moringa oleifera	Leaves	41.76	41.11	30.86
	Stem	45.46	43.89	29.46
Khaya senegalensis	Leaves	45.46	41.01	31.66
	Stem	45.46	44.14	43.76
Negative Control		0.00	0.00	0.00
Diminazene Aceturate		13 µg/ml	4 µg/ml	1 µg/ml
		45.46	43.76	36.03

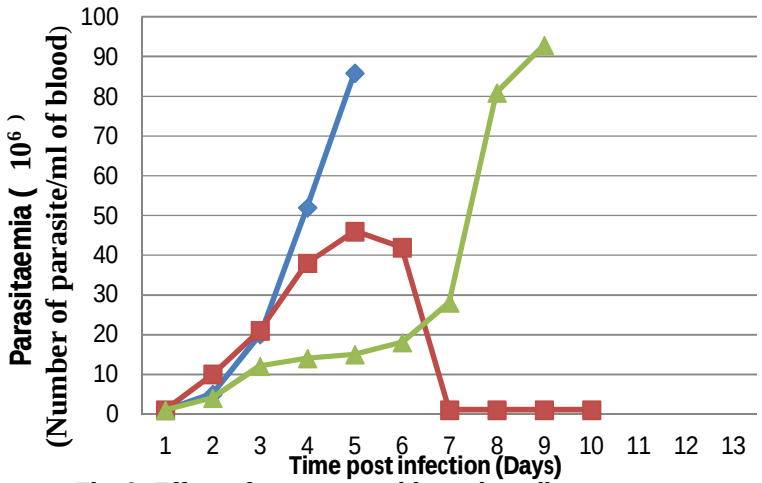

Fig. 2: Effect of treatment with methanolic extract on mean parasitaemia of mice infected with *T. brucei brucei* .
Khaya senegalensis **(▲); *Moringa oleifera* stem (■); Control (♦)**

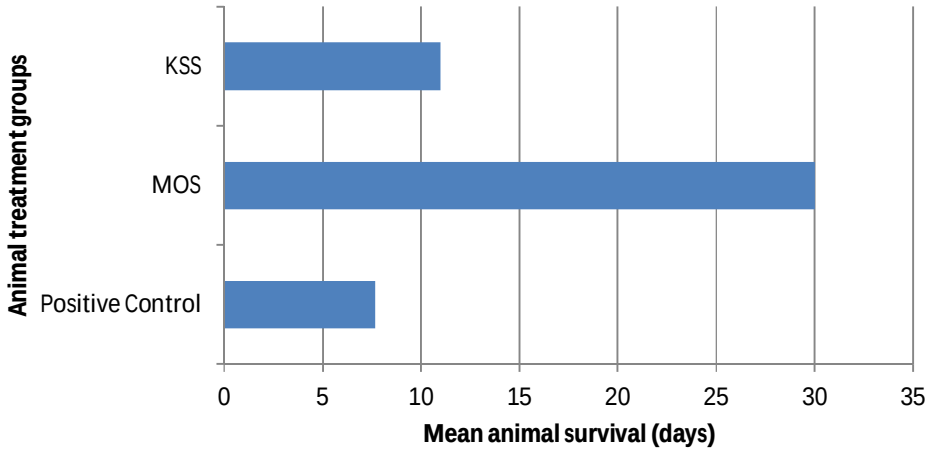

Fig. 3: Effect of treatment with methanolic extract of *Khaya senegalensis* stem (KSS) and *Moringa oleifera* stem (MOS) extracts on mean mice survival

Table 2. Infectivity results of mice subinoculated with different organ homogenates from *Moringa oleifera*-treated mouse

Days Post- Infection	Infectivity status (Number of infected mice/Number of total mice inoculated)			
	organ			
	Spleen	Kidney	Brain	Liver
1	0/3	0/3	0/3	0/3
2	0/3	0/3	0/3	0/3
3	0/3	0/3	0/3	0/3
4	3/3	2/3	1/3	1/2*
5	3/3	3/3	2/3	2/2
6	3/3	3/3	3/3	2/2

*AD indicates accidental death of mouse.

DISCUSSION

Different concentrations of the methanolic extracts of stems and leaves of Khaya senegalensis and Moringaoleifera were studied for their in vitro and in vivo antitrypanocidal activities against T. brucei brucei parasite. Decrease in the per cent population of motile parasites when compared with untreated control was taken as a measure of in vitro trypanocidal activity of extracts. The methanolic extract of Khaya senegalensis was found to possess the highest in vitro activity with the percentage mortality of 43.76 % at lowest concentration of 300 µg/ml. The least activity was found in Moring aoleifera stem (29.46 % Mortality). This observation compared well with that of diminazene aceturate, the standard trypanocidal drug employed as a control during the experiment, whose mechanism of action has been well established (Gutteridge and Coombs, 1977). Extracts from Moringa oleifera stem and leaves had a little effect on the parasite during the two hours of incubation. This variation could be partly attributed to differences in type and amount of phytochemicals in the various parts of the plants.

When the two most active extracts from Khaya senegalensis and Moringa oleifera stems were tested for possible in vivo antitrypanosomal potential, the result obtained revealed the suppression of parasitaemia by the methanoloic extract of Khaya senegalensis stem and near-total elimination of blood parasite by Moringa oleifera extract and these were accompanied by equivalent pattern of animal survival. This seems, somewhat, to support the claim that plants with high in vitro antitripanosomal activity may have no in vivo activity and vice versa (Asuzu et al.,1990; Nok et al 1993 ; Ibrahim et al 2008). Peculiarities in the plants' constituents could partly explain such findings (Umar et al., 2010). This results corroborates the need to subject in vitro results to further in vivo scrutiny. Again, during any in vivo chemotherapeutic screening of synthetic or plant-derived antitrypanosomal agents, organs from treated animals should be subjected to histopathological investigation even if there were no circulating parasite in the blood after treatment.

In the subinoculation experiments, it was observed that all the mice treated with various tissue homogenates from Liver, Spleen, Kidney and Brain showed high parasitaemia ten days post subinoculation. This revealed that though the parasites had been cleared from the blood, they were still present within the cellular compartment of the treated animal. Higher doses, say 500 mg/kg b.w., would have, perhaps, cleared the remaining parasites from these tissues.

Of particular interest in the result of the subinoculation experiment, is the presence of trypanosomes in the brain long after they have been cleared from the blood. It has been established that the late stage of African trypanosomiasis is neurologic in nature and is accompanied by the presence of the parasite in the brain. This means that the parasite is able to cross the blood brain barrier (BBB) in attempt to evade chemotherapy and so any successful trypanocidal compound must also have functional groups that permit its passage across the BBB. It is also possible, in addition to being in low concentration in the tissues, that the trypanocidal principles in Moringa oleifera stem extract lacked the physico-chemical property that could have allowed passage into the brain. However if coupled to a blood transporter like transferin, it is possible to have its antitrypanocidal potential enhanced. This approach has been demonstrated by Nok and Nock (2002) using azanthraquinone. Another possible approach that could improve the antitrypanosomal activity of Moringa oleifera extract is administering it along side another extract that can cross the BBB. In this case, Moringa oleifera stem extract will eliminate the circulating parasites quickly making the elimination of trypanosomes within the brain tissues easier even with lower concentrations of the other drug component.

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