NJBAS
Available online at http://www.ajol.info/browse-journals
Nigerian Journal of Basic and Applied Science (2009), 17(2):257-264
ISSN 0794-5698

Studies on the *In Vitro* Trypanocidal Effect of the Extracts of Some Selected Medicinal Plants in Sokoto State, Nigeria

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ABSTRACT: The *in vitro* trypanocidal activity of aqueous extracts of some selected medicinal plants used by local herdsmen in the treatment of various animal diseases in Sokoto State, Nigeria was conducted. *Trypanosoma brucei brucei* were cultured using 96 well micro titer plate and maintained at 37^0C. About 20 – 25 parasites per microscope field were dosed with 1, 2 and 4mg/ml of aqueous extracts of the plants and a control group without extracts. After 5 minutes incubation in Eppendorf tubes maintained at 37^0C, the parasites survived more than four (4) hours in the absence of extract/Berenil. At 4mg/ml of the extracts 0f *Terminalia catappa, Waltheria indica, Cucurbita pepo, Entada abyssinica* and *Ximenia Americana,* complete cessation of motility of *T. brucei brucei* within 60 minutes was observed. However, at 2mg/ml of *Waltheria indica,* trypanosome motility after 20 minutes was stopped but *Terminalia catappa,* and *Ximenia americana* were found to reduce trypanosome motility at 35 and 55 minutes respectively. Only *Waltheria indica* reduced trypanosome motility within 25 minutes at 1mg/ml concentration. Berenil, the standard drug, caused cessation of trypanosomal motility within 60 minutes even at 1mg/ml. From the results *Waltheria indica* was the most effective among the extracts when compared with Berenil, and may be a potential source of compounds with trypanocidal activity.
KEYWORDS: *in vitro, trypanocidal activity, medicinal plants, sokoto, Nigeria*

INTRODUCTION

Human Trypanosomiasis is endemic in Africa and South America. In Africa, the disease known as Human African Trypanosomiasis (HAT) or sleeping sickness, is caused by *Trypanosoma brucei gambiense* (chronic form) or *T. brucei rhodesiense* (acute form), whereas the American Trypanosomiasis known as Chagas' disease, is caused by *T. cruzi*. Sleeping sickness and Chagas' disease are both transmitted by vectors (Segura *et al.,* 1999). Human African Trypanosomiasis is transmitted during blood meals by tsetse flies, while the American Trypanosomiasis is transmitted by reduviid bugs. In addition to human infectious trypanosomes, a variety of other species cause animal Trypanosomiasis with a wide geographic distribution. *Nagana* is caused by *T. brucei brucei* in Africa and affects cattle; *T. congolense* and *T. vivax* infect domestic and small animals on many continents (Hoare, 1972). *T. brucei brucei* and *T. congolense* are similar to *T. brucei gambiense* and *T. brucei rhodesiense*, in that they are transmitted through tsetse fly bites, while *T. evansi* is spread by mechanical transmission of infected blood through haematophagus insects such as tabanid flies. *T. evansi* is closely related to other *Trypanozoon* species, including *T. brucei gambiense* and *T. brucei rhodesiense*, at the generic level.

Trypanosomiasis has recently become resurgent in Africa (WHO, 1998). WHO reports that 66 million people in 36 African countries are afflicted and Animal Trypanosomiasis causes the death of 3 million cattle each year (WHO, 1998, Truc, 2003, Chreten and Smoak, 2005).

Chemotherapy remains the principal means of control (FAO, 1998) and possibly eradication of the disease (Ogbunugafor *et al.*, 2007, Ekanem and Oluwatosin, 2008). Current treatment of African Trypanosomiasis is problematic especially for patients with late stage disease and nervous system involvement. The available drugs cannot be taken orally and are generally toxic (TDR, 2005). Berenil is widely used for the treatment of the early stage animal Trypanosomiasis but is highly toxic (TDR, 2005), producing brain damage, quivering, and restlessness in treated animals, besides being excreted quickly (Kellner *et al.*, 1985).

There are only two drugs known to be effective against the late stage of the disease, Difloromethyl ornithine (DFMO, eflornithine®) and Melarsprol®. DFMO can only cure *T.*

brucei gambiense infections. Furthermore, because of the lengthy infusion schedules, it can only be administered in a hospital setting (Fijolek *et al*, 2007); it remains expensive to manufacturers (TDR, 2005) and is no longer available in the market (Agbedahunsi *et al*, 2006). Melarsprol, an arsenical derivative, has serious side effects such as fatal encephalophathy in as high as 10% of the cases. Furthermore, there is an increasing resistance to Melarsprol reaching almost 30% in Central Africa (Fijolek *et al.*, 2007).

Because of the highly variable nature of the trypanosome glycoprotein coat, all attempts to develop an efficient vaccine have met with little success (Fijolek *et al.*, 2007). These limitations in chemotherapy of sleeping sickness are a cause for major concern because the disease is 100% fatal (Agbedahunsi *et al.*, 2006).

Several reports on the evaluation of different chemicals/drugs for trypanocidal activity have appeared, just as are interesting reports on the anti*T.*l effects of plant extracts and plant derivatives (Atawodi *et al*, 2003). Some of these reports have indeed shown that at least some of these plants possess trypanocidal activity. Since herbal treatment for various diseases in Africa is still wide spread, an ethno-botanical approach in collaboration with traditional healers may prove to be a rich source of drug discovery. Moreover, Sokoto State, being a semi – arid region, is included in the tsetse free belt of the country and therefore left out in the current fight against Trypanosomiasis. But the zone is equally endowed with medicinal plants that may probably have the potential to cure the disease. In addition, Ajagbonna *et al.* (2003) and Bala *et al*. (2005) have shown Garlic and *Cassia occidentalis*, to produce a life prolonging effect on trypanosome – infected rats when treated with the plants.

Therefore, as a follow up to this, we present in this study, report on systematic *in vitro* assessment of aqueous extracts of some medicinal plants, found in Sokoto State, for their trypanocidal activity using *T. brucei brucei* as test organisms. It is hoped that results from the study will be of great importance in the current global effort to find alternative, cheap and effective products for the treatment of trypanosomiasis.

MATERIALS AND METHODS

Collection and Identification of the Plants

The choice of plants used in this study, was based on a structured oral interview conducted amongst the local herdsmen in different parts of Sokoto State. The herdsmen were asked the plants they use in treating animals diseases with debilitating effects. Some of these diseases are usually characterized by inappetence, lethargy, weakness, loss of weight, emaciation, pica, rough hair coat, lameness, abortion in females, infertility in male and female animals, salivation, lacrimation, etc which usually culminate in death if untreated.

The plants were identified at the Botany Unit of the Department of Biological Sciences, Usmanu Danfodiyo University, Sokoto. A voucher specimen for each plant was deposited in the herbarium of the Department (Table 1).

Table 1: Scientific name, English name, Local name, part of the plant used and local method of preparation of the selected medicinal plants

S/N.	Scientific name	English name	Local name	Part used	Local method of preparation and administration
1	*Cissus quadrangularis*	Edible stemmed vine	Daddoori, Dodoriya	Stem bark	Cold water infusion, oral
2	*Cucurbita pepo*	Pumpkin	Kabushi	Seeds	Boiled water extract, oral
3	*Entada abyssinica*	Splinter bean Abyssinia entada, Tree entada	**T**awatsa	Stem bark	Boiled water extract, oral
4	*Holarrehna floribunda*	Connesi, False rubber tree, holarrhena	Gamon sauwa	Stem bark	Cold water infusion, oral
5	*Stylosanthes erecta*	Nigerian stylo, Stylo, Tropical luceme	Tsira fakoo	Stem bark	Boiled water extract, oral
6	*Terminalia catappa*	Tropical almod, Fruiting Umbrella tree.	Baushe	Stem bark	Boiled water extract, oral
7	*Waltheria indica*	Sleepy morning	Hankufa	Whole plant	Cold water infusion, oral
8	*Ximenia americana*	Wild plum (Plum)	Tsada, Chabbuli	Stem bark	Cold water infusion, oral

EXTRACTION OF PLANTS MATERIALS

Whole part of *Waltheria indica*, seeds of *Cucurbita pepo* and stem barks of *Cissus quadrangularis*, *Holarrehna floribunda*, *Terminalia catappa*, *Entada abyssinica*, *Ximenia americana* and *Stylosanthes erecta* were cut into pieces, air-dried (under shade or in open air in the laboratory to avoid denaturing the active components) at room temperature and pulverized using mortar and pestle to coarse powder.

A 100g powder of each plant was extracted with 500ml of distilled water. It was then manually shaken vigorously for six hours (alternatively shaken for ten minutes and resting for 15 minutes). It was then allowed to stand for the next 18 hours, shaken again for ten minutes and filtered using size 1 Whatmann filter paper. The volume of each filtrate was noted and placed in an electric drier and evaporated slowly at 45^0C as described by Muyibi *et al.,* (2000).

TEST ORGANISMS

T. brucei brucei were obtained from stabilates maintained at the Nigerian Institute of Trypanosomiasis and Onchocerciasis Research (NITOR) Vom, Plateau State. The parasites were maintained in the laboratory by continuous passage in rats until required. Passage was carried out when parasitaemia reached between 16 – 32 parasites per field (usually 3 – 5 days post infection). In passaging, 1 x 10^3 parasites in 0.1 – 0.2ml blood/PBS solution was injected intraperitoneally into naive rats acclimatized under laboratory condition for one week.

DETERMINATION OF PARASITAEMIA

Parasitaemia was monitored in blood obtained from the tail, pre-sterilized with methylated spirit. The number of parasites was determined microscopically at X 400 magnification using the "Rapid Matching" method of Herbert and Lumsden (1976). Briefly, the method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with buffered phosphate saline (PBS, pH 7.2). Logarithmic values of these counts obtained by matching with the table of Herbert and Lumsden (1976) is converted to antilog to provide absolute number of trypanosomes per ml of blood.

IN VITRO TRYPANOCIDAL ACTIVITY

Assessment of *in vitro* trypanocidal activity was performed in triplicates in 96 well micro titer plates (Atawodi *et al*, 2003), but with slight modification. 0.5g of each extract was dissolved in 25ml distilled water to obtain 20mg/ml extract. Concentrations of 10 and 5mg/ml were also formed, giving three different concentrations for each extract. The 20, 10 and 5mg/ml concentrations of each plant extract were prepared in triplicates.

Twenty microlitres (20µl) of blood containing about 20 – 25 parasites per field obtained as described above, were mixed with 5µl of each extract to produce effective test concentrations of 4mg/ml, 2mg/ml and 1mg/ml respectively. To ensure that the effect monitored was that of the fraction alone, two sets of control were set up. First Diminazene aceturate (Berenil – a standard drug) was used as positive control and then blood suspended in normal saline was used as a second (negative) control. Berenil was prepared in the same concentration as the extracts (445mg Diminazene aceturate + 555mg Antipyrine, Eagle Chemical Company Ltd, Ikeja, Nigeria)

Each of the test mixtures was incubated for 5 minutes in closed Eppendorf tubes maintained at 37^0C. Two(2) µl of test mixture was placed on separate microscope slides and covered with a cover slip and the parasites observed every 5 minutes for a total duration of sixty (60) minutes. Cessation or drop in motility of the parasites in extract-treated blood compared to that of the parasite-loaded control blood without extract was taken as a measure of trypanocidal activity (Atawodi *et al*, 2003).

RESULTS

The percentage yield of the various plants extracts is shown in Table 2. Five plants, namely *Cucurbita pepo, Entada abyssinica, Terminalia catappa, Waltheria indica* and *Ximenia americana* caused complete cessation of motility of *T. brucei brucei* within 60 minutes at 4mg/ml extract concentration (Table 3). *Waltheria indica* caused the cessation within the first 10 minutes, *Terminalia catappa* and *Ximenia americana* caused cessation within 55 minutes, while *Cucurbita pepo* and *Entada abyssinica* stopped trypanosome motility within 60 minutes. Two plants, *Stylosanthes erecta* and *Holarrehna floribunda* caused slight reduction in motility of *T. brucei brucei* within 50 and 60 minutes respectively. The extracts of *Cissus quadrangularis* had no effect on the parasites at this concentration.

Table 2: Percentage Yields of Extracts

S/NO	Plant	Yield (%)
1.	*Cissus quadrangularis*	27.24
2.	*Cucurbita pepo*	22.60
3.	*Entada abyssinica*	16.80
4.	*Holarrehna floribunda*	21.18
5.	*Stylosanthes erecta*	23.72
6.	*Terminalia catappa*	26.53
7.	*Waltheria indica*	21.75
8.	*Ximenia Americana*	19.36

Table 3: Effect of Different Concentrations of the Aqueous Extracts of Some Medicinal Plants on Motility of *T. brucei brucei*

	Time (Min) after which motility ceased, reduced drastically, or reduced slightly with different concentrations of extracts (mg/ml)		
Plant	4	2	1
Cissus quadrangularis	-	-	-
Cucurbita pepo	30^{SRM} 40** 60*	40^{SRM} 60**	-
Entada abyssinica	25^{SRM} 45** 60*	-	-
Holarrehna floribunda	60^{SRM}	-	-
Stylosanthes erecta	50^{SRM}	-	-
Terminalia catappa	10^{SRM} 20** 55*	25^{SRM} 35**	55^{SRM}
Waltheria indica	5** 10*	5^{ARM} 15** 20*	15^{SRM} 25** 55*
Ximenia americana	25^{SRM} 40** 55*	45^{SRM} 55**	-
Diminazene aceturate	5*	5*	5** 15*

- = No effect on motility after 60 minutes, SRM = slightly reduced motility, * = ceased/stopped motility, ** = reduced motility drastically.

At 2mg/ml *Waltheria indica* caused stoppage in trypanosome motility after 20 minutes, *Terminalia catappa* and *Ximenia americana* drastically reduced trypanosome motility after 35 and 55 minutes respectively, while at this concentration, the other plant extracts had no effect on the parasites.

At 1mg/ml concentration, *Waltheria indica* drastically reduced trypanosome motility after 25 minutes, while all other plant extracts had no such effect on the trypanosome parasites.

Diminazene aceturate (Berenil) caused cessation of *T.*l motility within 6o minutes even at the lowest concentration tested (1mg/ml).

The results also showed that after 5 minutes incubation in Eppendorf tubes maintained at 37^0C, the trypanosome parasites survived for about 4 hours when no extract was present.

From the results *Waltheria indica* was the most effective plant extract at all concentrations tested. The result also indicated that the extract of *Waltheria indica* was the most effective at 4mg/ml concentration because it stopped trypanosome motility within 10 minutes. At same concentration Berenil caused cessation of movement after 5 minutes.

DISCUSSION

Results of this study showed that the extracts of some of the plants (*Terminalia catappa*, *Waltheria indica, Cucubita pepo*, *Entada abyssinica* and *Ximenia americana*) had strong trypanocidal activity *in vitro*, while the extracts of *Stylosanthes erecta* and *Holarrhena floribunda* had moderate trypanocidal activity, with extract of *Cissus quadrangularis* showing no *in vitro* effect.

That some of the plants showed promising trypanocidal effect is not surprising, since earlier reports (Asuzu and Chineme, 1990; Owolabi *et al*, 1990; Nok *et al*, 1993; Freiburghaus *et al.*, 1996, 1997, 1998; Bala *et al*, 2005; Agbedahunsi *et al*, 2006) have clearly indicated that plants of different families could possess potent trypanocidal activity. In fact, natural products with trypanocidal activity and belonging to a variety of phytochemical classes have been identified (Hopp *et al*, 1976; Sepulveda – Boza and Cassels, 1996; Oliver – Bever, 1986).

The reported *in vivo* trypanocidal activity of the extract of *Terminalia spp* at 150mg/kg in mice experimentally infected with trypanosome parasites in Mali (Bizimana *et al*, 2006), is confirmed by this *in vitro* assay.

In an earlier report (Bacchi, 2003), the leaf extract of *Holarrhena floribunda* was shown to possess *in vivo* trypanocidal activity in trypanosome infected mice. The present report of lack of *in vitro* trypanocidal activity of the stem bark extract emphasises the need to study all parts of a plant before any generalisation is made on the plant therapeutic potential.

Moreover, a plant with high *in vitro* trypanocidal activity may have no *in vivo* activity and vice versa. This is because there are peculiarities in the metabolic disposition of the chemical constituents of plants. Therefore plants found to be active in this study must be tested *in vivo* before a definite statement can be made on their trypanocidal potentials.

It is difficult to speculate the mechanism by which the plants extracts' exhibit their trypanocidal action. However accumulated evidence (Sepulveda – Boza and Cassels, 1996) suggest that many natural products exhibit their trypanocidal activity by virtue of their interference with the redox balance of the parasites acting either on the respiratory chain or on the cellular defences against oxidative stress. This is because natural products possess structures capable of generating radicals that may cause peroxidative damage to trypanothione reductase that is very sensitive to alterations in redox balance. It is also known that some agents act by binding with the kinetoplast DNA of the parasite.

The findings in this study clearly reaffirm the *in vitro* system employed in this investigation as a fast and reliable system for *in vitro* screening of plants and other materials for trypanocidal activity.

From this brief study, it seems clear that local herdsmen do indeed possess a noteworthy knowledge of medicinal plants which should be acknowledged and considered in the search for novel compounds for the treatment of parasitic diseases. Further studies including *in vivo* assays for the determination of efficacy and toxicity are recommended.

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