

In Vitro, In Vivo and Phytochemical Screening, of Extracts of Piper guineense for Trypanocidal Activities Against Trypanosoma brucei brucei.

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Abstract

Extracts from leaves and stem bark of *Piper guineense* were evaluated for trypanocidal activity against *Trypanosoma brucei brucei* in vitro and in vivo. The phytochemical screening of the most active extracts in vitro was carried out. The Petroleum ether extract of stem bark exhibited highest in vitro trypanocidal activity out of the six extracts tested. Thus it was used to treat animals infected with *Trypanosoma brucei brucei* at a dose range of 50-200 mg/kg body weight, intraperitoneally and orally. The results showed that the group treated with 100 mg/kg body weight intraperitoneally showed significant reduction in parasitemia with a prolonged life span up to the 18th day post infection. While those that were treated with 200 mg/kg body weight orally, had progressive increase in parasitemia up to the 7th and 8th day post infection when they all died. However in the prophylactic experiment animals that were treated with 50 mg/kg intraperitoneally and 100 mg/kg orally for five days before challenging them with 10³ trypanosomes, did not develop parasitemia up to 28th day post infection when the experiment was terminated. This experiment shows that the petroleum ether of *Piper guineense* possesses some degree of trypanocidal activity and further purification of the active component should be carried out.

Keywords: *Piper guineense*, phytochemical screening, *Trypanosoma brucei brucei*, in vitro and in vivo activity

1. Introduction

Trypanosomiasis is a parasitic disease caused by trypanosomes, they are unicellular parasites transmitted by the bites of tsetse fly, it is the causative agent of sleeping sickness in humans and related diseases in animals, which is a major factor retarding the growth of livestock industry in Africa (Brun et al., 2001). It is estimated that some 35 million people and 25 million cattle in Africa are at the risk of the disease (WHO, 2004). One of the important pathogenic trypanosomes in animal is *Trypanosoma brucei*, the causative agent of sleeping sickness in humans and commonly known as Nagana in domestic animals which is highly fatal to some cattle, sheep and goats (Motelmams, 1986; ILARD, 1996). Despite the high prevalence rate, only a few drugs, namely Suramin Pentamidine, Melarsoprol and eflorintine are in use. However there usage is beset with problems including limited repertoire of compound, toxicity, inherent expensive nature of current trypanocides, protracted treatment protocols and drug resistance (Legros et al., 2000; Kamuanga, 2003).

Furthermore the search for vaccination against African Trypanosomiasis remains elusive because of exhibition of antigenic variation (Kuzoe, 1993; Doua & Yapo, 1993; Donald, 1994). With all these problems mentioned it has become imperative to seek trypanocides that are cheaper, readily available and less toxic. Recent approach to alternative therapeutics agents for treatment of Trypanosomiasis have focused on plants and other natural products (Freiburghaus, 1996; Atawodi, 2003; Nok & William, 1996). *Piper guineense* commonly known as black pepper belongs to the family *piperaceae*. It has more than 700 species throughout the tropical and subtropical regions of the world. It is known with different vernacular names in Nigeria: Igbo (Uziza), and Yoruba (iyere). *Piper guineense* has culinary, medicinal, cosmetic and insecticidal uses (Dalziel, 1955; Okwute, 1992). The leaves are considered aperitive, carminative and eupeptic. (Sumathykutty et al., 1999).

They are also used for the treatment of cough, bronchitis, intestinal diseases and rheumatism. These effects have been attributed to the peptides, alkaloids, essential oils, phenols and flavonoids which are major components in these plants (Okigbo & Igwe, 2007).

2. Materials and Methods

2.1 Plant Collection

The plant materials of *Piper guineense* were collected from Zaria metropolis of Kaduna state and taken to the Department of Biological Sciences, Ahmadu Bello University Zaria for identification.

The leaves and the stem bark were dried at room temperature before crushing into powder, using pestle and mortar. It was then stored separately in air tight containers and kept at 4°C until needed.

2.2 Preparation of Crude Plant Extract

Fifty grams of each dried grounded plant material was defatted in 200 ml of Petroleum ether, in a round bottom flask with reflux condenser. The whole mixture was mounted on a shaker and be allowed to macerate for 10 hrs. The extract was filtered using whatman No.1 filter paper and the filtrate concentrated by distillation in a rotary evaporator at 50°C. The resultant residue of fats waxes and steroids was collected separately and stored in specimen bottles in a refrigerator at 4°C until required. The mac recovered from the Petroleum extract was air dried at room temperature (36°C) and refluxed exhaustively in 200 ml methanol for 10 hrs, using the same procedure described earlier. 5 ml of glacial acetic acid was added to the methanolic filtrate before evaporation to prevent the loss of active component (Nok et al., 1993). The same procedure was performed with aqueous extract using 200 ml of distilled water.

2.3 Experimental Animals

All animals used were healthy albino mice of both sexes. They were purchased from the animal house of Nigerian Institute for Trypanosomiasis Research, Kaduna (NITR). They were about ten to twelve weeks old, weighing on the average 22-25 g. The animals were kept in well ventilated clean, plastic cages, they were fed with commercial chick grower's mesh (ECWA feeds, Jos, Nigeria) and Water was given *ad libitum*.

2.4 Parasites

A Stabilate of pleomorphic *Trypanosoma brucei brucei* was obtained From Nigerian Institute for Trypanosomiasis Research Kaduna and maintained by serial passage in Swiss albino mice which served as donor animal.

2.5 Determination of Parasite

Parasitemia was monitored daily in infected animal microscopically from blood obtained from the tail, at x 40 magnification. The rapid matching method described by Herbert and Lumsden (1976) was used to estimate the number of parasite per field. Briefly, parasites were counted under the microscope per field either in pure blood or blood appropriately diluted with Phosphate buffered saline. The obtained value was matched with the logarithm table of Herbert and Lumsden (1976) and was then converted to antilog, thus absolute number of trypanosomes per ml of blood.

2.6 In Vitro Trypanocidal Activity

Two donor mice were sacrificed and their blood was collected in heparinised tubes for *in vitro* assessment. Trypanocidal activity was performed in duplicates in 96 well micro titre plate (Flow laboratories Inc., McLean, Virginia 22101, USA). Ten milligram of the various extract were weighed and dissolved in 1ml of phosphate buffer saline separately and were further diluted serially to obtain effective concentration ranging from 2.5-10 mg/ml. The blood obtained from the donor mouse containing 30-35 trypanosomes was dispensed into a solution of glucose phosphate buffer saline in the ratio of 1-2. Of which fifty micro litres was dispensed into a well of the micro titre plate mixed with 20 µl of the constituted extract to give a final volume of 70 µl. Test concentration of 2.5 mg, 5 mg, and 10 mg/ml. A set of control was included which contained the parasite suspended in phosphate buffer saline only. For reference, tests were also performed with the same concentrations of *Diminal^R* (445 mg *diminazene diacetate*+ 555 mg phenazone/g, Eagle Chemical Company LTD, Ikeja, Nigeria) a commercial trypanocidal drug. After 5 min incubation in covered micro titre plate maintained at 37°C, a drop of the test mixtures were placed on separate microscope slides and covered with cover slips and the parasites observed every 5 minutes for a total duration of two hrs. It should be noted that under this *in vitro* system adopted, parasites survived for about 4h when no extract was present. Cessation or drop in motility of the parasites in extract-treated blood compared to that of parasite-loaded control blood without extract was taken as an indication of trypanocidal activity.

2.7 BIIT

A blood incubation infectivity test was performed, as follows: parasite suspension incubated in the presence of the petroleum ether extract of stem bark of *Piper guineense* at 2.5 mg, 5 mg and 10 mg/ml, as described for *in vitro* studies. After an incubation period of 2 hrs, 0.2 ml of each preparation was injected in duplicate into healthy mice and the level of parasitemia was assessed as described earlier for two weeks.

2.8 Phytochemical Screening

Chemical test was carried out on the powdered specimen of methanolic leaf extract and petroleum ether extract of stem bark of *Pipe guineense*, using standard procedure to identify the constituents as described by (Odebisi & Sofowora, 1978). This is to identify the presence of tannins, resin, glycosides, flavonoids, alkaloids, saponins among others.

2.9 Acute Toxicity Study

Sixteen albino male mice (weighing 20-25 g) of both sexes were purchased from the animal house of Nigerian Institute for Trypanosomiasis Research Kaduna (NITR). They were randomly divided into four groups (ABCD) of four mice each and kept in clean cages. They were feed with pellets and water ad libitum. Group A, B & C were administered graded doses (50, 100, 500 and 1000 mg/kg) of the petroleum extracts of the stem bark of plant intraperitoneally while animals in group E were given normal saline only.

After administration of the extracts (0.2 ml in mice intraperitoneally) the animals were observed continuously for 72 hrs and 7 days for any signs of behavioural changes, toxicity and mortality. Mortality of animals during the period was recorded. The LD⁵⁰ of the extracts were calculated by the method of Lorke et al. (1983). The control group received vehicle alone.

2.10 Prophylactic Activity of Stem Bark Petroleum Ether Extract

The test for prophylactic activity was done as described by Igwe and Onabanjo 1989 with slight moderation. Thirty mice were divided into six groups (A, B, C, D, E, & F) of five mice each. Groups A and B received a dose of 20 and 50 mg/kg of the stem bark extracts intraperitoneally each, while oral administration at 50 and 100 mg/kg were given to groups C and D respectively for five consecutive days. Group E and F served as positive and negative control respectively. On the sixth day, they were challenged with 10³ trypanosomes. Infected mice were then routinely monitored microscopically for parasitemia from tail blood smear as described earlier, for twenty one days.

2.11 In Vivo Antitrypanosomal Activity of Crude Extract of *Piper guineense*

Thirty mice were divided into six groups (A, B, C, D, E, and F) of five each. They were infected with 10³ trypanosomes each, the level of parasitemia was monitored daily by the Herbert and Lumsden method (1976). At the peak of parasitemia such as 25-35 per field as described under "determination of parasitemia", groups A and B were given 50 and 100 mg/kg body weight of the constituted extract respectively through intraperitoneally route. While 100 and 200 mg/kg of the constituted extract were administered to groups C and D orally respectively. All animals were treated for seven consecutive days. Except for groups E and F which served as control, they were infected not treated but were given normal saline intraperitoneally and orally respectively.

3. Results

The extracts obtained from various parts of *Piper guineense* showed different time of *in vitro* motility activity against *Trypanosoma brucei brucei* at different concentrations (Table 1). The petroleum ether extract of Stem bark of *Piper guineense* showed highest activity in a dose dependent fashion. Low *in vitro* activities were observed with both aqueous extract of leave and stem bark. Furthermore, there was no activity with extracts of Petroleum ether leaf and methanolic stem bark of *Piper guineense* at the end 120 minutes. However Diminal^R eliminate parasite motility in all concentrations (20-50) minutes of incubation.

The blood incubation infectivity test showed that when these parasites suspensions were incubated with the extract at 2.5, 5, and 10 mg/ml, parasite motility was completely ceased at 120 minutes. Also, when sub inoculated into healthy mice, the suspensions with 2.5 mg and 5 mg/ml concentration of extract came down with parasitemia between the 4th and the 6th days, while animals inoculated with suspension of 10 mg/ml concentration of extract did not come down with parasitemia.

The toxicological studies showed that the extract produced dose related death in mice 0/5, 50 mg/kg; 1/5, 100 mg/kg; 2/5, 500 mg/kg and 4/5, 1000 mg/kg. The LD⁵⁰ was calculated to be 250 mg/kg with 95% confidence limits of 115-335 mg/kg.

The prophylactic activity showed that administering extract for five days (Figure 1) to mice at 50 mg/kg and 100 mg/kg intraperitoneally and orally to groups (B&D) respectively did not lead to the development of parasiteamia. However the groups that were given 20 mg/kg intraperitoneally and 50 mg/kg orally (A&C) came down with the disease on the 6th and 4th day respectively.

Similarly the *in vivo* result presented in (Figure 2), showed that there was great reduction in parasiteamia with group (B) treated with 100 mg/kg intraperitoneally. Which led to their life's been prolonged up to the 18th day post infection compared to the infected control in group E which increased progressively until the death of the animals between days sixth and seventh post infection. furthermore, group A, treated with 50 mg/kg intraperitoneally, while groups C and D treated orally with 100 and 200 mg/kg body weight respectively also cumulated into death by the 9th day post infection.

Table 1. Trypanocidal activity of different concentration of extract from parts of *piper guineense*

Plant name	Plant part	Time in minutes which motility ceased reduced slightly reduced drastically with different effective concentrations of extracts (mg/ml)									
		Petroleum ether			Methanol			Aqueous			
		10	5	2.5	10	5	2.5	10	5	2.5	
<i>Piper guineense</i>	Leaves	10	5	2.5	10min***	30min***	60min**	80min*	NA	NA	
		NA	NA	NA							
<i>Piper guineense</i>	Stembark	5min**	10min***	25min***	NA	NA	NA	75min*	NA	NA	

NA= parasite highly motile after 120min, ***=motility ceased completely, **= motility reduced drastically, *= slightly reduced.

Table 2. Phytochemical screening of methanolic leave and Petroleum ether stem bark extract of *Piper guineense*

part of plant	extract	Alkaloids	Saponins	Cardiac glycosides	Anthraquinones	Terpinoids	Phlobatanins	Tannins	Sterols
leaves	Methanol	+	+	-	-	-	-	+	-
stem bark	Pet ether	++	+	-	-	+	-	+	-

++ = highly present, + = moderately present, -- = absent.

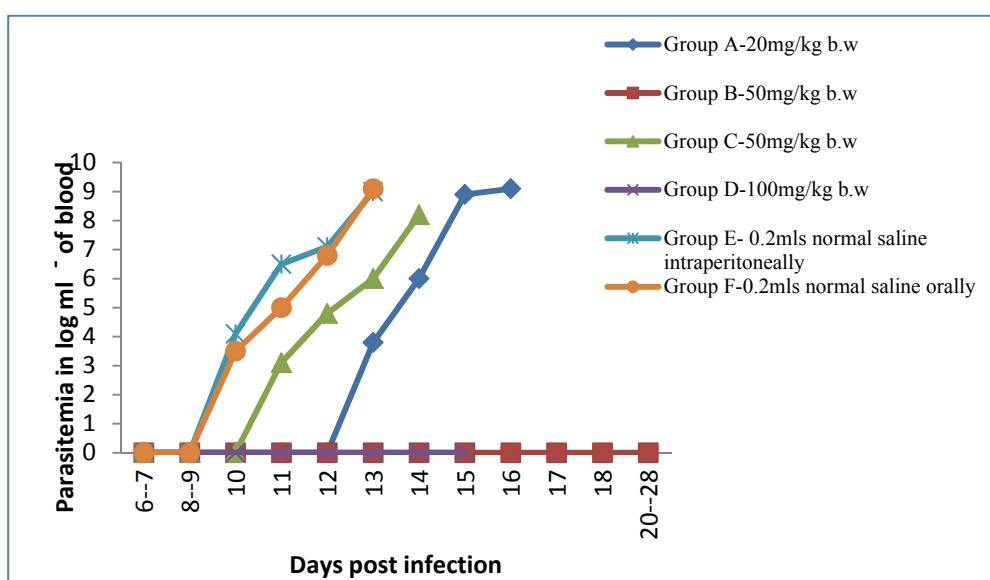


Figure 1. Effect of administration of pet-ether extract of *Piper guineenses* to mice for 5 consecutive days before been challenged with parasite on the sixth day

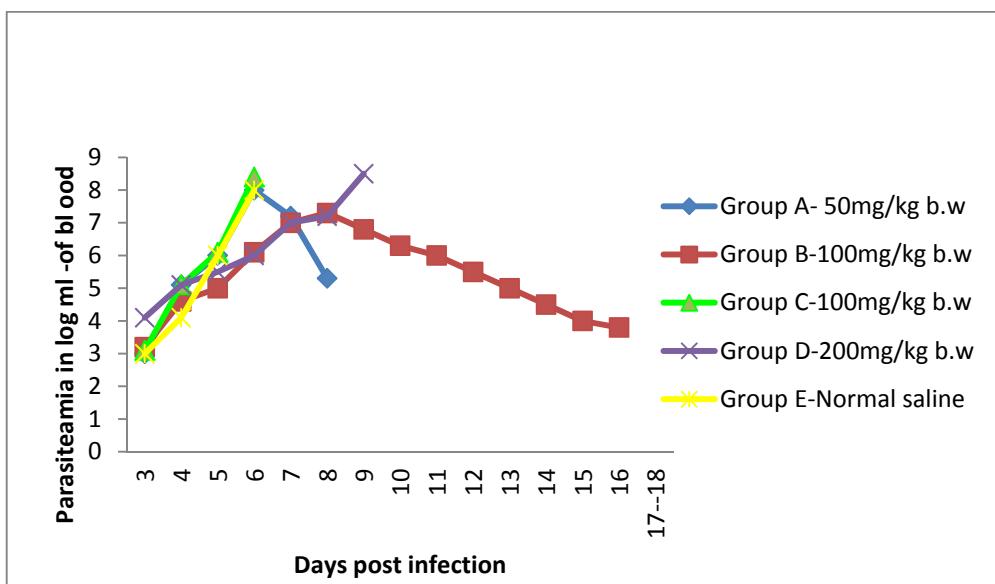


Figure 2. Course of parasitemia in five groups of mice treated with extract of stem bark of *Piper guineense* for seven days

4. Discussion

The result of the *in vitro* studies showed clearly that the antitrypanosomal activity vary with different parts of the plant material. Although the stem bark of *Piper guineense* exhibit the highest activity out of the extracts tested. This investigation supports earlier reports which indicate that different parts of plant of the same plant may exhibit different activity (Freiburghaus, 1996; Atawodi et al., 2003) due to composition and concentration in different chemical components. Hence drop in motility of trypanosomes could therefore be used as an indication of Trypanosomal potentials of crude extract when compared with the control parasite motility and this could be taken as indication for trypanosomal activity (Nok, 2001; Atawodi et al., 2002). The *in vivo* experiment was conducted to support the evidence of efficacy of the methanolic extract *in vitro*. There was appreciable decline in parasitemia especially in animals treated intraperitoneally with 100 mg/kg (Group B) compared with those treated with 200 mg/kg orally (Group D). Also the culmination of death of animals even at low levels of parasitemia, may be connected to the findings of (Nwagwu et al., 1987; Boitigon et al., 1990) which shows that extracellular factors release by the trypanosomes have pathological effect on the host animal. Furthermore the toxins release into the mammalian system renders the antibodies produced by the host against the parasite to undergo antigenic variations, by changing the surface glycoprotein (Sternberg, 2004). However the prophylactic result exhibit a very interesting result, in that parasitemia did not develop in animals treated with the extract at 50 mg/kg intraperitoneally and 100mg/kg orally for five days before been challenged with the parasite. This result gave a far more significant trypanocidal effect than that observed with the same dose of extract used for curative treatments. Which suggest that the plant extract possess compounds with trypanocidal properties that can be further investigated. The Phytochemical screening (Table 2) showed that the stem bark extract contains an appreciable amount of alkaloids and tannins. Although previous report attributed the trypanocidal activity of a number of tropical plants to alkaloids, saponins, babarine (Nok et al., 2001).

In view of these findings and the quest to source for trypanocides from natural product we recommend further purification of the extracts which might led to pure compound and in turn give better *in vivo* result.

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