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In vitro and *In vivo* Antitrypanosomal Effects of *Mitracarpus villosus* Leaves

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ABSTRACT
The *in vitro* antitrypanosomal activity of the aqueous, ethanolic, methanolic and chloroform extracts of the leaves of *Mitracarpus villosus* was evaluated on *Trypanosoma b. brucei* by means of a blood incubation infectivity test (BIIT) using Diminal® (diminazene aceturate) as positive reference.. The aqueous extract was fractionated to yield eight fractions by means of a column chromatography, each of the eight fractions were tested *in vitro* by means of BIIT. The therapeutic potentials of fractions II, III, IV and the crude aqueous extract were investigated in mice infected with *T. brucei*; the animals were treated orally at doses of 100 and 200 mg/kg body weight daily until their death. Phytochemical screening of the aqueous extract showed the presence of flavonoids, tannins, alkaloids, anthraquinones, saponins, and glycosides. Fractions II, III and IV emerged as the only fractions with appreciable trypanocidal activity. Although, all the fractions and the crude aqueous extract showed some degree of activity at both doses, fraction IV conferred the best antitrypanosomal effect as it extended the life span of the animals by 4 days, when compared to the negative control, and its ability to decrease the parasites 8 days post treatment. Gas chromatography-mass spectrometry of fraction IV, revealed 13 peaks, two of which were 9, 10-Anthracenedione-α anthraquinone and (4-Phenyl-tetrahydro-pyran-4-ny)-piperidin-1-yl-methanone, derivatives of anthraquinones and alkaloids. The result revealed that the aqueous extract and fraction IV possessed trypanocidal activity which may be attributed to the presence of the anthraquinone and alkaloidal derivatives

Key words: *Mitracarpus villosus, Trypanosoma brucei,* anthraquinones, alkaloids, fractions, antitrypanosomal activity

INTRODUCTION

African trypanosomiasis has continued to threaten human health and economic development (Kuzoe, 1993; WHO, 2000). It causes human sleeping sickness and livestock trypanosomiasis in sub-Saharan Africa. Trypanosomes, the causative parasites are prevalent in Africa and has been responsible to a great extent for the under development, poverty and suffering in many parts of Africa (Holmes, 2000). The search for vaccination against African trypanosomiasis remains elusive and effective treatment is beset with problems of drug resistance and toxicity (Gutterridge, 1985; Aldhous, 1994; Onyeyili and Egwu, 1995). Four drugs (suramin, pentamidine, melarsoprol and eflornithine) are currently available to treat trypanosomiasis (Kuzoe, 1993), with only melarsoprol and eflornithine being effective against the meningoencephalitis that develops in the late stages of the disease. In addition to emerging cases of drug resistance, all the four drugs require lengthy, parenteral administration and all but eflornithine have severe toxic side effects (Gutterridge, 1985; Onyeyili and Egwu, 1995) thus, underscoring the urgent need to develop more effective and safer trypanocidal drugs. Several reports on the evaluation of different chemicals/drugs for trypanocidal activity have appeared (Bodley and Shapiro, 1995; Bodley *et al.*, 1995) just as are interesting reports on the antitrypanosomal effects of plant extracts and plant derivatives (Asuzu and Chineme, 1990; Nok *et al.*, 1993; Sepulveda-Boza *et al.*, 1995; Freiburghaus *et al.*, 1996, 1997, 1998; Atawodi *et al.*, 2003). Some of these reports have indeed shown that, at least under in vitro conditions, some of these plants possess trypanocidal activity (Freiburghaus *et al.*, 1996, 1997, 1998). Furthermore, several plant extracts or plant derivatives have been investigated *in vivo* for

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the antitrypanosomal efficacies in mice (Asuzu and Chineme, 1990; Nok 1992; Youan *et al.*, 1997).

Mitracarpus villosus is a medicinal plant whose decoction is used by some Northern Nigerian Fulani herdsmen for treatment of livestock trypanosomiasis (*Nagana*) in their cattle. However, no scientific proof has been laid to ascertain this claim, although, Irobi and Daramola (1993, 1994) have earlier reported the anti-fungal and bactericidal effects of this plant. It is against this background that the present investigation was conducted to primarily justify the acclaimed efficacy of *M. villosus* in curing livestock trypanosomal infections among the Fulani herdsmen of Northern Nigeria.

MATERIALS AND METHODS

Materials

Seventy, healthy mice were obtained from the Department of Animal Science, National Veterinary Research Institute (NVRI), Vom, Jos, Nigeria; while *T. b. brucei* was obtained from the Department of Veterinary Parasitology, University of Nigeria Nsukka, Nsukka, Enugu State, through passage in mice. *M. villosus* was collected within Zaria, Kaduna State and identified at the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria. All the reagents used were of analytical grade.

Extraction procedure

Portions (100 g) of the powdered shade-dried plant were each extracted using 500 ml of distill water, ethanol, methanol and chloroform for 24 hours. The filtrates obtained were concentrated to dryness by evaporation. The extracts were stored at 4°C in tightly sealed containers till needed.

Phytochemical screening of extracts

All the four extracts were screened for the presence of phytochemicals as described by Harborne (1973) and Trease and Evans (1989).

In vitro trypanocidal activity of the crude extracts of Mitracarpus villosus

A blood incubation infectivity test (BIIT) was performed as described by Atawodi *et al* (2003) and Nok *et al* (1992). Trypanosome-infected blood samples were incubated in 96 well micro-titer plates in the presence of the extracts. Exactly 0.1 g of the extracts each were dissolved in 5 mls of distilled water or 0.5% dimethyl sulfoxide (DMSO) to give a stock solution of 20 mg/ml, and from this stock, other concentrations were made by dilution (10 and 5mg/ml); phosphate buffer saline glucose (PBSG) was also added to provide the requisite energy source for the parasites now in an *in vitro* environment. Each extract was diluted in three-fold serial dilutions after which each concentration was tested in triplicates. A reference drug (Diminal®) was also prepared at various concentrations of 20, 10, and 5mg/ml. Control wells containing the infected blood with the buffer without plant extracts were included as well as control wells with the 0.5% DMSO. The incubated blood samples with the crude extracts were checked at 5 minutes interval for one hour for the motility of the parasite in the blood using a microscope at x40 magnification.

Partial purification of plant extract (Column chromatography)

The crude extract was partially purified using column chromatography. Briefly, a slurry was prepared by shaking 200 g of silica gel (Merek 70-230 mesh powder S.A) with 400 mls of ethyl acetate, methanol and water (EMW) in the ratio 40:10:1 and packed in a column. The column was then loaded with 15 g of the crude aqueous extract that had shown the highest potency in the *in vitro* test as described previously in the present study. It was eluted with the solvent system (EMW) in the same 40:10:1 ratio. Eight fractions were collected and evaporated to dryness to obtain 0.19, 0.36, 0.16, 0.43, 0.17, 0.10, 0.18 and 0.16 of extracts designated as eluted fractions I-VIII.

Second phase of blood infectivity incubation test

In vitro anti-trypanosomal activities of the various fractions was determined as described previously at the concentrations of 20, 10 and 5 mg/ml.

Animal handling, infection and grouping

The adult male mice were allowed to acclimatize for two weeks in the animal house of the Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria after which the mice were randomly assigned into thirteen groups (n = 5). Food and water were provided

without restrictions. Blood was collected by cardiac puncture with an ETDA coated syringe from a heavily infected rat and immediately diluted with physiological saline to serve as inoculums. Animals in Groups 2-11 were infected intraperitoneally with 0.01 ml of the inoculums, such that about 1 X 10^5 parasites/100g were injected into the animals. Infection was then monitored every morning by microscopic examination of blood sample taken from the tails of infected animals. Group 1 was uninfected and served as normal control while animals in groups 12 and 13 were not infected but treated with the crude extract at 100 and 200 mg/kg body weight, respectively. Animals in Group 2 were treated with a reference drug (Diminal®) at the dose recommended by the manufacturer, serving as positive control, while those in group 3 was untreated and thus served as the negative control. Rats in Groups 4 and 5 were treated with fraction II at 100 and 200 mg/kg body weight, respectively; groups 6 and 7 with fraction III, while those in groups 8 and 9 were treated with fraction IV at the same doses of 100 and 200 mg/kg body weight respectively. Groups 10 and 11 were treated like those of groups 8 and 9 except they received the crude aqueous extract.

Determination of parasitaemia

Parasitaemia was monitored in the blood obtained from the tail, pre-sterilized with methylated spirit. The number of parasites was determined microscopically at a magnification of x400 using the "Rapid Matching" method described by Herbert and Lumsden (1976).

Determination of packed cell volume (PCV)

PCV was determined every three days on all the 65 mice using the microheamatocrit method using blood collected from the tail of the animals.

Monitoring the life span

The life span after infection and treatment was recorded for all the sixty five mice. This was done by observing all groups and recording the mortality in any of the groups in the mornings and evenings throughout the experimental period.

Gas chromatography - mass spectrophotometry (GC-MS) analysis

The GC-MS analysis of the fraction that showed the best anti-trypanosomal activity *in vitro* and *in vivo* was performed using a *GCMS-QP2010 PLUS SHIMADZU*, Japan gas chromatograph equipped with an Elite-5 capillary column.

RESULTS AND DISCUSSION

The phytochemical screening of the plant leaves confirmed the presence of carbohydrates, anthraquinones and glycosides in all the extracts, while flavonoids, saponins and tannins were found in the aqueous, methanolic, and ethanolic extracts, but absent in the chloroform extract (Table 1). This difference can be attributed to the solubility of the active components in the different solvents (Ekpo and Etim, 2009).

The aqueous extracts from *M. villosus* leaves showed a complete cessation of the motility of *Trypanosoma brucei brucei* (*T. brucei*) within 60 minutes (Figure 1). Variation in anti-trypanosomal activities has been observed in instances where plant extracts from different solvents were tested (Wurochekke and Nok, 2004). Freiburghaus *et al* (1996) opined that solvent extracts of the same plant may exhibit different trypanocidal activity, just as extracts of different parts of the same plant. The aqueous extract of the leaves eliminated the parasites completely within 20 minutes at the highest extract concentration (20 mg/ml) when compared with the other extracts. Atawodi *et al* (2003) reported that the shorter the time of cessation of motility of the parasite, the more active the extract; this formed the rationale for chosing the aqueous extract for subsequent investigation in the present study. In addition, Diminal® eliminated trypanosomal motility completely at 35 minutes at a dose of 20 mg/ml. Other research findings reported that Diminal® can eliminate the parasite even at lower doses (Atawodi *et al*., 2003).

Since the crude extract of *M. villosus* leaves showed a promising trypanocidal effect against the *T. brucei* parasite, it was further purified using Column chromatography where eight fractions were collected and tested for their trypanocidal effect *in vitro*. Fractions (II, III and IV) were found to have prominent anti-

trypanosomal activity against *T. brucei*, (Figure 2).

Table 1: Phytochemical constituents of *Mitracarpus villosus*

Extracts	Alkaloids	Anthraquinones	Carbohydrates	Cardiac Glycosides	Flavonoids	Steroids	Saponins	Tannins	Triterpenes
Aqueous	++	++	+++	+	+++	_	+	++	+
Ethanolic	_	+	+	+	_	_	+	+	_
Methanolic	_	+	+	+	+	_	+	+	_
Chloroform	_	+	+	+	_	_	_	_	_

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

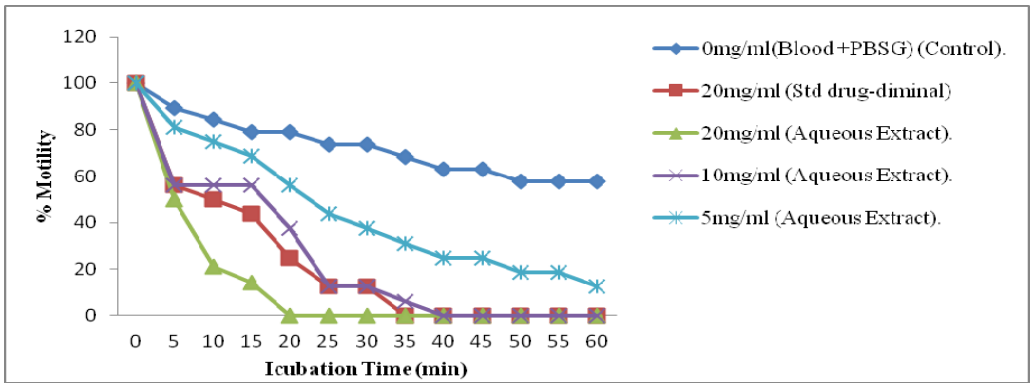

Fig. 1: Percentage motility of the parasites against time by aqueous extract of *M. villosus* leaves

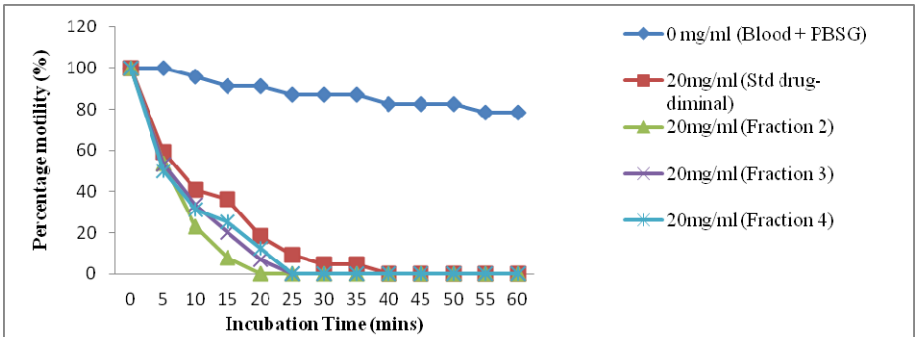

Fig. 2: Percentage motility of the parasites against time by fractions II, III and IV

This might be due to the presence of some effective phytochemical components in the fractions such as flavonoids, alkaloids and anthraquinones which have been reported previously to posses some trypanocidal effects (Nok, 2002; Tarus *et al*., 2002; Sara *et al*., 2003).

Fractions II, III, IV and the crude aqueous extract also exhibited some anti-trypanosomal activity *in vivo* (Figure 3a & 3b). However, only fraction IV at 200 mg/kg body weight and the crude extract at 100 mg/kg body weight possessed the most effective anti-trypanosomal activity. It is difficult to speculate the mechanism by which fractions IV and the crude extract of *M. villosus* leaves exhibited this anti-trypanosomal activity. However, accumulated evidence suggests that many natural products exhibit their trypanocidal activity by interfering with the redox balance of the parasites acting either on the respiratory chain or on the cellular defenses against oxidative stress (Atawodi *et al*., 2003; Maikai *et al*., 2008). Furthermore, phytochemicals, in contrast to the synthetic pharmaceuticals, based upon single chemicals, may exert their effects through additive or synergistic action of several chemical compounds acting at a single or multiple target sites associated with a physiological process (Kaufmann *et al*., 1999). In addition, it has also been reported that some agents act by binding with the kinetoplast DNA of the parasite (Atawodi *et al*., 2003).

The failure of the fractions and crude extract to protect the animals from anemia induced by the parasites (Figure 4) could be due to either the route of administration, absence of some elements in the plant extract needed for blood production, detoxifying actions of phase I and phase II mechanisms in the liver, enzymatic inactivation of the active compounds from the gut or reduced absorption from the gut or a combination of two or more of the mentioned reasons. Furthermore, the present observation suggests a possible treatment failure as observed by Legros *et al* (1999), who reported that treatment failure is possible in cases of massive parasiteamia at the time of the therapeutic intervention. It is possible that early treatments with higher concentrations of fractions II, III, IV and the crude aqueous extract may have conferred treatment success as illustrated in the *in vitro* results in Figure 2, or may have served effectively in a prophylactic capacity.

To obtain information on the type of compounds which could be responsible for the anti-trypanosomal activity in fraction IV, Gas Chromatography-Mass Spectrometry (GC-MS) analysis was conducted which revealed 13 peaks (Table 2); amongst which were 9, 10-Anthracenedione-α Anthraquinone, and (4-Phenyl-tetrahydro-pyran-4-ny)-piperidin-1-yl-methanone, which are derivatives of the anthraquinones and alkaloids respectively whose anti-trypanosomal effect in other plants have earlier been reported (Nok, 2002; Tarus *et al*., 2002; Sara *et al*., 2003).

In conclusion, extracts of *Mitracarpus villosus* leaves showed appreciable anti-trypanosomal activity *in vitro*. However, the aqueous extract exhibited the highest anti-trypanosomal activity whereas fractions IV among all the fractions showed the most effective anti-trypanosomal activity. This study confirms the claim by the Fulani herdsmen in using the decoction of the plant leaves in treating "*Nagana"* in their animals.

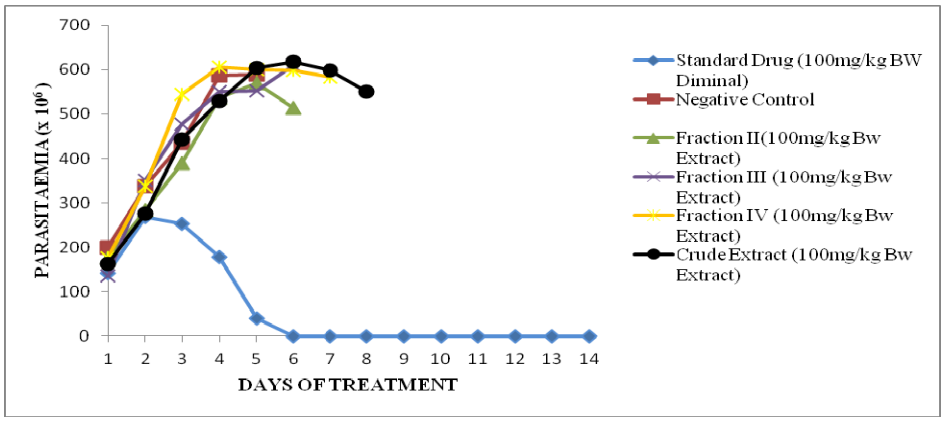

Fig. 3a: Effects of administration of Fractions II, III, IV and the crude aqueous extract of *M. villosus* on 100 mg/kg body weight per day *T. brucei* infected mice.

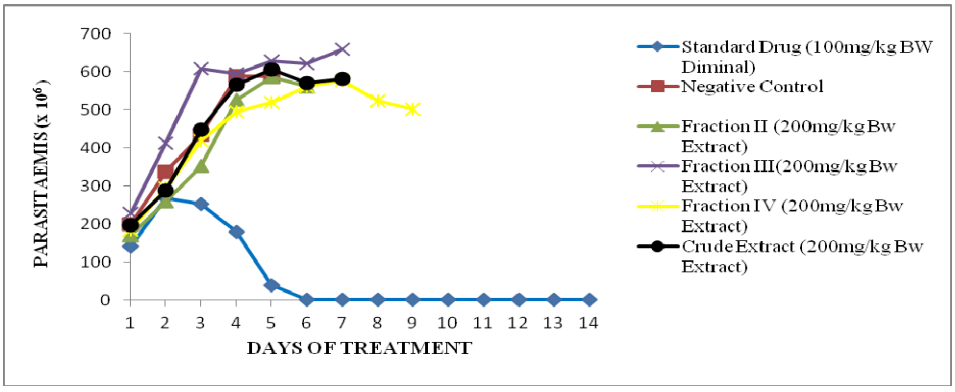

Fig. 3b: Effects of administration of Fractions II, III, IV and the crude aqueous extract *M. villosus* at 200 mg/kg body weight per day on *T. brucei* infected mice.

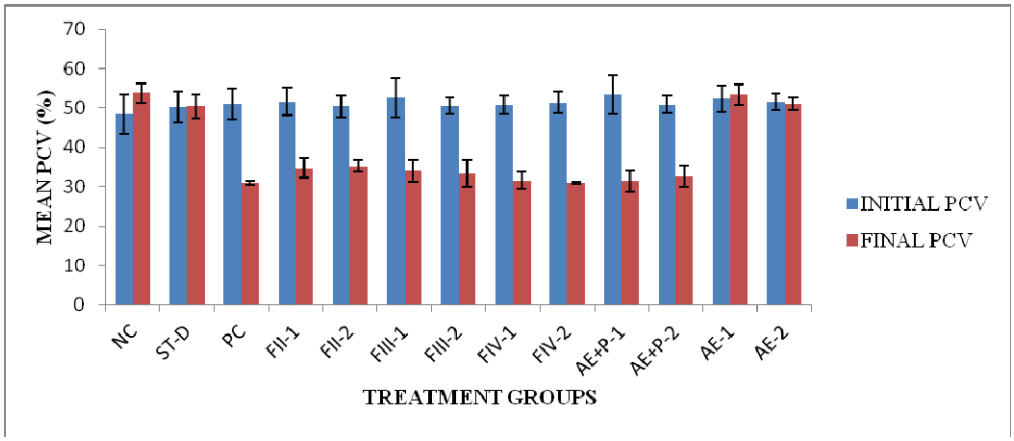

Fig. 4:Changes in packed cell T. *brucei brucei* infected mice by Fractions II, III, IV and the crude aqueous extract (100 and 200 mg/kg body weight/day) of *M. villosus*

NC=Normal Control, ST-D= Standard drug control, PC= Parasite (Negative) control, FII-1= Fraction II at 100 mg/kg body weight, FII-2= Fraction II at 200 mg/kg body weight, FIII-1= Fraction III at 100 mg/kg body weight, FIII-2= Fraction III at 200 mg/kg body weight, FIV-1= Fraction IV at 100 mg/kg body weight, FIV-2= Fraction IV at 200 mg/kg body weight, AE+P-1= Aqueous extract infected control treated at 100 mg/kg body weight, AE+P-2= Aqueous extract infected control treated at 200 mg/kg body weight, AE-1= Uninfected aqueous extract control treated at 100 mg/kg body weight, AE-2= Uninfected aqueous extract control treated at 200 mg/kg body weight.

Table 2: GC-MS constituents of Fraction IV

Peak	Compound Name	Formular	Molecular weight	Retention Time (mins)	Base peak	Similarity Index (%)
1	Methyl 3-butynoate	$C_5H_6O_2$	98	15.2	97.90	75
2	α-L-Galactopyranoside	$C_7H_{14}O_5$	178	18.4	60.05	83
3	9, 10-Anthracenedione-α Anthraquinone	$C_{14}H_8O_2$	208	31.73	208.20	84
4	Pentadecanoic acid	$C_{15}H_{30}O_2$	242	32.16	73.00	85
5	α-2-Hexadecen-1-ol	$C_{20}H_{40}O$	296	33.79	71.05	90
6	9,12,15-Octadecatrien-1-ol	$C_{18}H_{32}O$	264	33.97	79.00	80
7	9,9-Dimethoxybicyclo[3.3.1]nona-2,4-dione	$C_{11}H_{16}O_4$	212	34.23	73.00	76
8	Hexadecanoic acid	$C_{19}H_{38}O_4$	330	37.06	97.95	84
9	3,6-Dimetyloxazolo(5,4-c)pyridazin-4-amine	$C_7H_8N_{40}$	164	39.22	164.40	68
10	[2-(4-propionylamino-furazan-3-yl)-benzoimidazol-1-yl]-acetic acid ester	$C_{16}H_{17}N_5O_4$	343	40.95	314.15	52
11	(4-Phenyl-tetrahydro-pyran-4-ny)-piperidin-1-yl-methanone	$C_{17}H_{23}NO_2$	273	41.63	111.85	61
12	Benzo(a)phenzine	$C_{16}H_{10}N_2$	230	42.8	230.52	56
13	4-(6-Methoxy-3-methyl-2-benzofuranyl)-3-buten-2-one	$C_{14}H_{14}O_3$	230	43.42	314.15	54

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