

Full Length Research Paper

# ***In vitro* and *in vivo* trypanocidal activity of *Combretum racemosum* leaves**

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**A study was conducted to determine the *in vitro* and *in vivo* trypanocidal activity of crude methanolic extract of *Combretum racemosum* leaves against *Trypanosoma brucei brucei*. The extract exhibited *in vitro* activity by immobilizing the trypanosomes and rendering them uninfected to mice at 125 to 0.2559 mg/ml. The extract also reduced parasitaemia and improved packed cell volume in infected mice at 50, 100 and 200 mg/kg body weight when administered intraperitoneally. Intraperitoneal administration of diaminazene aceturate to infected mice completely cleared the parasites from the blood. In addition, intraperitoneal administration of the extract to mice at 2,000 mg/kg body weight did not result in deaths during the acute toxicity study. This study therefore provides evidence of the ethnopharmacological use of *C. racemosum* in trypanosomiasis.**

**Key words:** *Combretum racemosum*, leaves, methanolic extract, trypanocidal activity, *Trypanosoma brucei*.

## **INTRODUCTION**

Trypanosomiasis is a severe often fatal disease endemic in sub Saharan Africa where it affects the general health and wellbeing of human and livestock populations (Igwe et al., 2002; Kamuanga, 2003). Since it has not been possible to develop an effective vaccine against the disease due to the problem of antigenic variation, trypanocidal drugs play a major role in its management and control (Igwe et al., 2002). The chemotherapy of Africa trypanosomiasis still remains far from being satisfactory and there is growing resistance to the few drugs currently available (De Koning, 2001; Ogbadoyi et al., 2007). Also, many of the drugs currently available for the treatment of trypanosomiasis are highly toxic (Matovu et al., 2001; Ogbadoyi et al., 2007).

*Combretum racemosum* P. Beauv (Combretaceae) is a straggling shrub widespread across Africa and bears a mass of crimson flowers which is very spectacular, giving it the local English name of Christmas rose in Southern Nigeria (Burkill, 1985). The leaf extract of *C. racemosum* is used in traditional medicine as an antiulcer (Okwuosa

et al., 2006), trypanocidal (Atindehou et al., 2004), antihelminthic and antibacterial agent for genito-urinary and gastrointestinal infections (Onocha et al., 2008). Previous phytochemical analysis of *C. racemosum* extracts revealed the presence of alkaloids, steroids, cardiac glycosides, saponins and tannins (Onocha et al., 2008)

The serious problem encountered in the control of both human and animal trypanosomiasis and the urgent need for new affordable trypanocidal drugs (Bizimana et al., 2006) suggests herbal remedies as a reasonable alternative. Literature surveys and field studies show that plants are used in traditional medicine in Africa to treat trypanosomiasis in humans and animals (Youan et al., 1997; Bizimana et al., 2006). The aim of this study was therefore to evaluate the trypanocidal activity of crude 70% methanol extract of *C. racemosum* leaves, both *in vitro* and *in vivo*.

## **MATERIALS AND METHODS**

Fresh leaves of *C. racemosum* were collected in April 2009 from Ngwo, Enugu state, Nigeria. They were identified by a taxonomist at the Department of Botany, University of Nigeria, Nsukka, where the voucher specimens are kept in the herbarium.

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### Preparation of extracts

The leaves were washed with water to remove dirt and dust, and then dried in the shade. The dried materials were ground into fine powder using a laboratory mill. Cold extraction was done with 70% methanol for 72 h at room temperature with intermittent shaking. After filtration through Whatman's filter paper, solvent was removed using rotary evaporator and the extract stored at -4°C in sterile universal tubes until use.

### Experimental animals

Albino mice weighing between 30 to 39 g of either sex procured from the Department of Veterinary Surgery, University of Nigeria, Nsukka, were used for the study. The animals were kept in clean wire meshed cages under standard animal house conditions in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (DHHS, NIH Publication No. 85-23, 1985). The mice were given standard pellet diet and water *ad libitum* during the entire period of experimentation.

### Parasite

The *Federe* strain of *Trypanosoma brucei brucei* was procured from the Nigerian Institute for Trypanosomiasis Research, Vom, Nigeria, where the initial stock was collected from a clinical case of bovine trypanosomiasis. The strain was maintained in the laboratory by successive serial passages in mice. For *in vivo* trials, whole blood (diluted with normal saline) from infected mice were inoculated intraperitoneally (IP) with a parasite inoculum of  $10^6$  trypanosomes per mouse. Trypanosomes in whole blood of infected mice were used for *in vitro* studies. Blood was collected from the retrobulbar plexus of the medial canthus of infected mice. The quantitative estimation of trypanosomes was done by rapid matching method (Herbert and Lumsden, 1976).

### Acute toxicity studies

Twelve mice were used for this study. The mice were divided into 4 groups (A to D) of 3 mice each. They were dosed intraperitoneally (IP) once with 250, 500, 1000 and 2000 mg/kg body weight of the extract, respectively. The mice were observed for 24 h for signs of toxicity, such as changes in behaviour or death.

### *In vitro* evaluation of the extract

The methanolic extract of *C. racemosum* leaves was tested using phosphate buffered Ringer's glucose solution as supporting medium according to a modified method of Petana (1964). Briefly, test tubes, each containing 1 ml of supporting medium, was used to prepare 2-fold serial drug dilutions (of the extract) covering a range from 125 to 0.0640 mg/ml. A final tube (also with 1 ml supporting medium) did not contain extract and served as the control. Blood stream forms ( $1 \times 10^6$ ) of *T. brucei* in 0.1 ml of the supporting media were added to each tube and incubated for 3 h at 37°C. The contents of each tube was examined microscopically half hourly for motility assessment.

The infectivity of the trypanosomes after incubation with extract was checked by intraperitoneal inoculation of the contents of each tube into 3 mice at a dose of 0.1 ml/mouse. Tail blood was collected daily from each mouse and checked for the presence of trypanosomes using the wet blood film and buffy coat methods (Woo, 1971). The abolition of infectivity of the parasite was concluded if no trypanosome was detectable for 60 days.

### *In vivo* evaluation of the extract

Thirty mice randomly divided into 6 groups (I, II, III, IV, V and VI) of 5 mice each were used for this study. Animals in groups I, II, III, IV and V were inoculated with  $10^6$  trypanosomes IP, while Group VI animals were not infected. After detection of parasitaemia (by day 6 post infection in all infected groups), animals in groups I, II and III received test extract at 50, 100 and 200 mg/kg body weight respectively by IP route daily for 5 consecutive days from day 12 post infection (PI). Animals in group IV were given diamminazene aceturate (Berenil<sup>®</sup>, Hoechst AG) at 7 mg/kg body weight IP on day 12 PI. Moreover, animals in groups V and VI did not receive treatment and served as positive and negative controls respectively. The parameters used to assess efficacy of the test extract included parasitaemia estimated by the rapid matching method (Herbert and Lumsden, 1976) and packed cell volume determined by the microhaematocrit method (Campbell and Coles, 1986). Initial detection of parasitaemia was from tail blood by the wet blood film and buffy coat method (Woo, 1971). Parasitaemia and packed cell volumes were determined at day 0 (onset) of the experiment and subsequently at 4-day intervals until termination of the study.

### Statistical analysis

The data collected were summarized as means  $\pm$  standard error of means. Statistical comparisons between the treatment groups were made by one-way analysis of variance (ANOVA). Means were considered significant at  $P < 0.05$  and the means separated using Duncan's multiple range test.

## RESULTS

### Plant extraction

The extract of *C. racemosum* leaves was dark brown in colour and pasty in consistency. The percentage yield was 8.1% (w/w).

### Acute toxicity study

There was no mortality recorded. However, some of the mice that received the extract at 1,000 and 2,000 mg/kg were weak and depressed for about 20 min after administration of the extract.

### *In vitro* activity of *C. racemosum* against *T. brucei*

The extract immobilized the parasites (Table 1) and rendered them uninfective to mice (Table 2) following incubation for 30 min at concentrations of 125 - 16.375 mg/ml. At 0.2559 mg/ml, trypanosomes were immobilized after 180 min. Mice inoculated with tube contents with extract concentrations of 125 - 0.2559 remained aparasitaemic for 60 days PI, while the control was parasitaemic 6 days PI.

### *In vivo* activity of *C. racemosum* against *T. brucei*

Trypanosomes were detected in the blood of infected

**Table 1.** Effect of *Combretum racemosum* leaf extract on motility of *Trypanosoma brucei brucei*.

Extract concentration (mg/ml)	Time (min)						
	0	30	60	90	120	150	180
125	+ve	-ve	-ve	-ve	-ve	-ve	-ve
65.5	+ve	-ve	-ve	-ve	-ve	-ve	-ve
32.75	+ve	-ve	-ve	-ve	-ve	-ve	-ve
16.375	+ve	-ve	-ve	-ve	-ve	-ve	-ve
8.1875	+ve	+ve	-ve	-ve	-ve	-ve	-ve
4.0937	+ve	+ve	-ve	-ve	-ve	-ve	-ve
2.0467	+ve	+ve	+ve	-ve	-ve	-ve	-ve
1.0234	+ve	+ve	+ve	+ve	-ve	-ve	-ve
0.5117	+ve	+ve	+ve	+ve	+ve	-ve	-ve
0.2559	+ve	+ve	+ve	+ve	+ve	+ve	-ve
0.1280	+ve	+ve	+ve	+ve	+ve	+ve	+ve
0.0640	+ve	+ve	+ve	+ve	+ve	+ve	+ve
0.0 (control)	+ve	+ve	+ve	+ve	+ve	+ve	+ve

-ve, Absence of motile trypanosomes; +ve, presence of motile trypanosomes.

**Table 2.** Effect of *Combretum racemosum* leaf extract on infectivity of *Trypanosoma brucei brucei* in mice.

S/N	Extract concentration (mg/ml)	Number of mice inoculated	infection/ parasitaemia	Survival of mice
1	125.0	3	N	S
2	62.5	3	N	S
3	32.75	3	N	S
4	16.375	3	N	S
5	8.1875	3	N	S
6	4.0937	3	N	S
7	2.0467	3	N	S
8	1.0234	3	N	S
9	0.5117	3	N	S
10	0.2559	3	N	S
11	0.1280	3	P	NS
12	0.0640	3	P	NS
13	0.0 (control)	3	P	NS

N, No. parasite detected; P, parasite observed; S, all mice survived the 60 days infectivity observation period' NS, none of the mice survived the 60 days infectivity observation period.

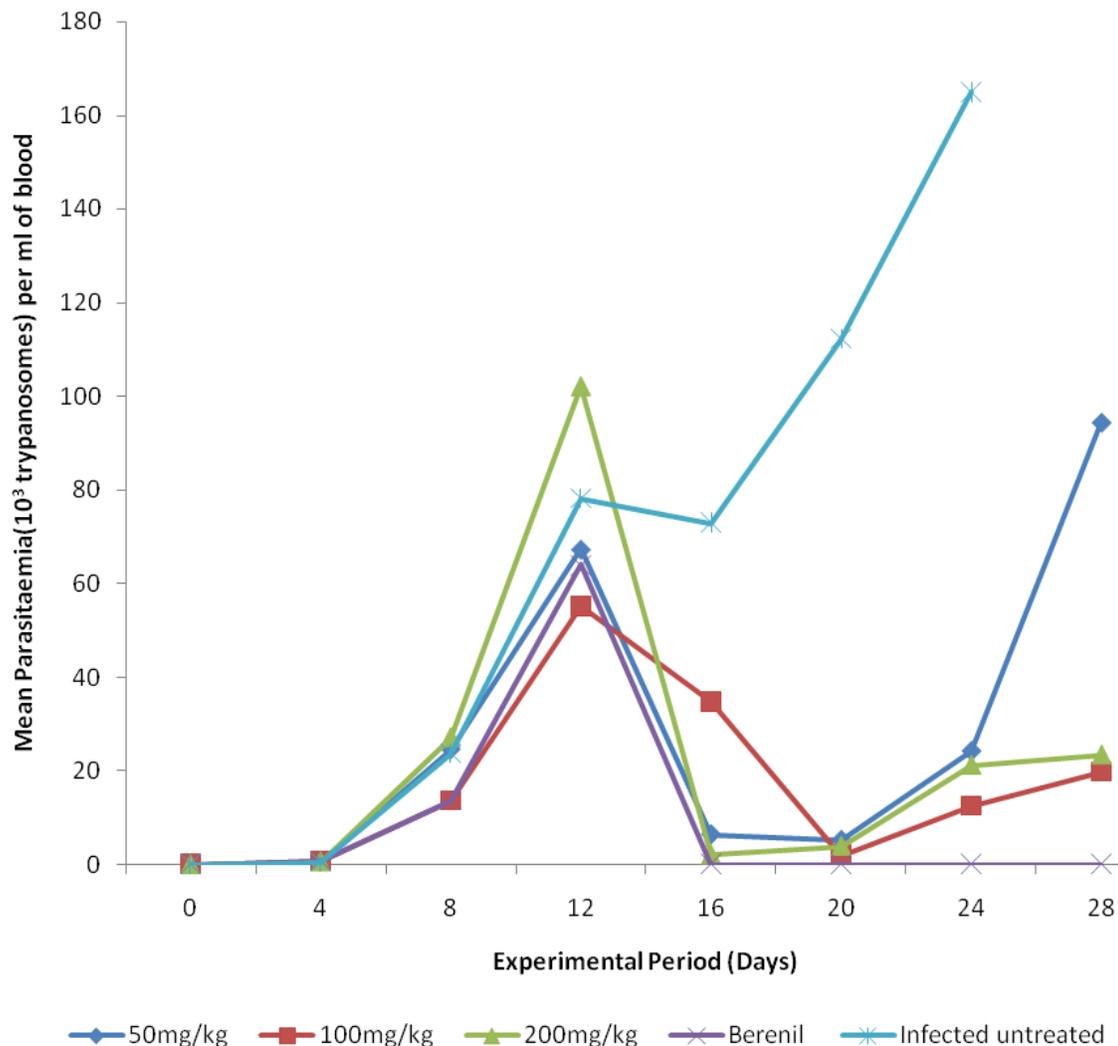
mice from day 4 PI. This was followed by progressive increase in mean parasitaemia in all the infected groups of mice (Figure 1). Treatment with the extract from day 12 PI caused significant ( $p < 0.05$ ) reduction in mean parasitaemia by day 16 PI when compared to the infected untreated group V. Diaminazene aceturate (group IV) cleared the parasites by day 16 PI. Meanwhile, resurgence in parasitaemia in the extract treated groups occurred by day 20 PI.

Following infection, the PCV in extract and diaminazene aceturate treated groups declined. This was followed by improvements after treatment (Figure 2). By day 16 PI, there was no significant difference ( $p < 0.05$ ) in PCV between the extract and diaminazene aceturate

treated groups, but by day 24 PI the PCV of the diaminazene aceturate treated group was significantly higher than the extract treated groups.

## DISCUSSION

The absence of death following administration of the extract at 2000 mg/kg showed that the extract was well tolerated. However, this result does not rule out possible cumulative toxic effects of administering the extract for 5 consecutive days as was done in the *in vivo* test. The result of the acute toxicity study was in agreement with previous work by Akubue et al. (1983) in which C.



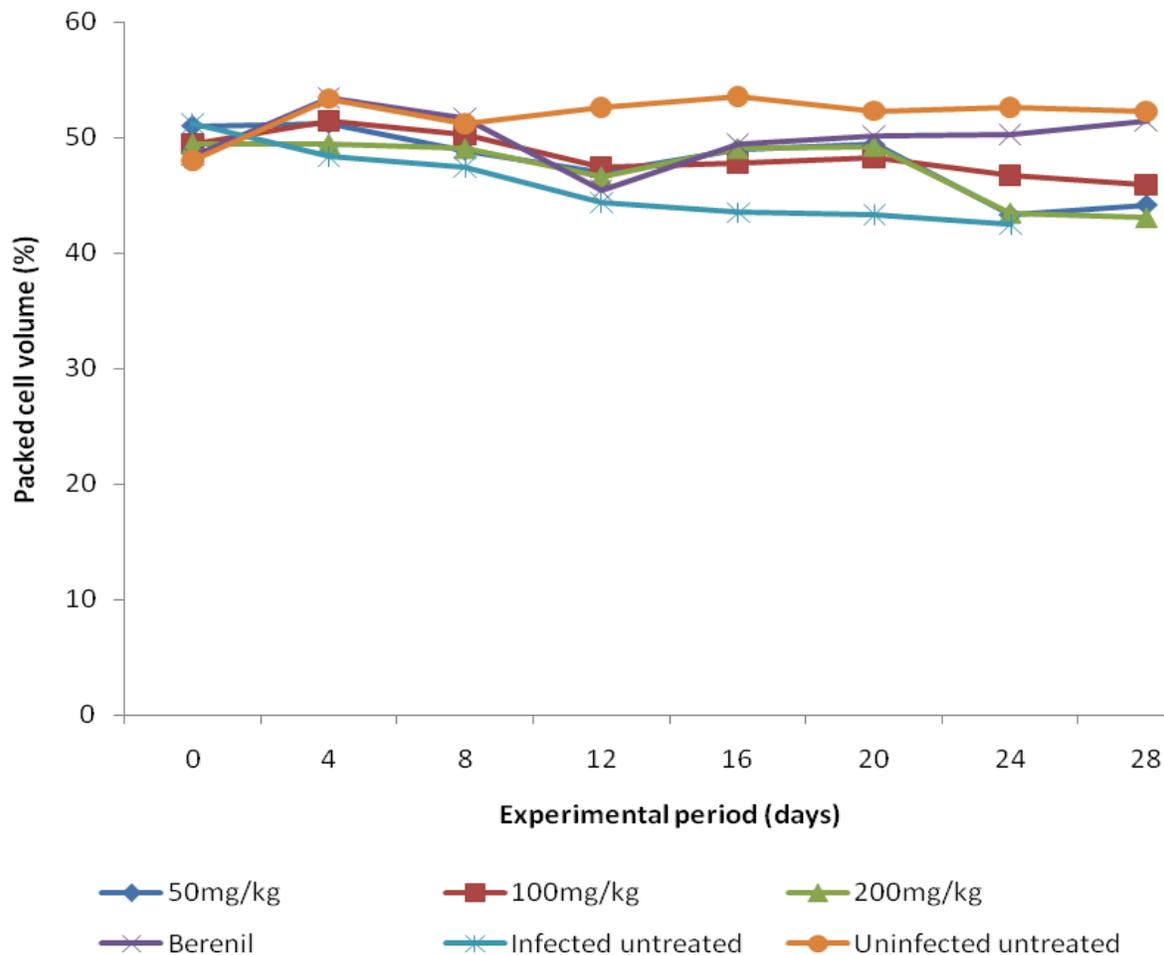
**Figure 1.** Mean parasitaemia of mice infected with *Trypanosoma brucei brucei* and treated with diaminazene aceturate and varying doses of *Combretum racemosum* extract.

*racemosum* leaf extract was found to have an intraperitoneal LD<sub>50</sub> of 17,780 mg/kg. Toxicity tests are employed in determining the possible dosages at which crude extracts can be administered to experimental animals. Arbitrary use of drugs/compounds in treatment without carrying out an acute toxicity test could be fatal (Nweze and Obiwulu, 2009).

In the *in vitro* study, the lowest concentration of extract which resulted in complete elimination of motility (minimum lethal concentration, MLC) was 0.2559 mg/ml. In addition, inoculation of mice with trypanosomes (from tube contents) with varying extract concentrations (125 to 0.2559 mg/ml) following an incubation period of 3 h did not result in infection. This demonstrates the *in vitro* trypanocidal activity of *C. racemosum* leaves, and it is also the first report on the trypanocidal activity of *C. racemosum*. Our results agree with previous studies (Wurochekke and Nok, 2004; Ogbadoyi et al., 2007;

Mbaya et al., 2010) that reported the *in vitro* trypanocidal activity of some medicinal plants. It is difficult to speculate the mechanism by which these 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 (Sepulveda-Boza and Cassels, 1996).

In the *in vivo* study, there was a reduction in parasitaemia following administration of extract in groups I-III mice. There was a significant difference in mean parasitaemia between the extract and the infected-



**Figure 2.** Mean packed cell volume of *Trypanosoma brucei brucei* infected mice treated with diaminazene aceturate and varying doses of *Combretum racemosum* leaf extract.

untreated group V mice at day 16 PI. This demonstrates the *in vivo* anti-trypanosomal activity of the extract. Treatment of the group IV mice with diaminazene aceturate (DA) led to the clearance of the parasites from the blood by day 16 PI, unlike the extract treated groups which had low levels of parasitaemia at day 16 PI. This shows that DA was more effective than the extract at the doses (50, 100 and 200 mg/kg) tested. Blood et al. (1994) has reported the curative effects of DA when used against *T. brucei* infection. This resurgence in mean parasitaemia in the extract treated groups, unlike in the DA group by day 20 PI may be due to the waning effect of the treatment. This resurgence in mean parasitaemia may also be due to release of trypanosomes from the tissues which can occur when treatment is delayed or the dose rate is inadequate (Blood et al., 1994).

Furthermore, infection of mice with *T. brucei* resulted in a decrease in mean PCV in all the infected groups. Anaemia is a consistent finding in trypanosomosis (Blood et al., 1994). Following administration of the extract or

DA, there was an initial improvement in PCV in all the treated groups, demonstrating the anti-trypanosomal activity of both the extract and DA. However, following resurgence of parasitaemia in the extract treated groups I, II and III mice at day 20 PI, there was a concomitant reduction in PCV values. This was in contrast to the DA group in which PCV continued to improve until it had returned to pre infection values by day 28 PI. Diaminazene aceturate was thus more effective than the extract in improving the PCV.

It can be concluded from the result of this study that although the crude extract of *C. racemosum* leaves possesses *in vitro* and *in vivo* activity against *T. brucei*, the extract was not effective enough to cure mice from parasitaemia. Better results may be achieved following phytochemical investigation with bioassay-guided isolation of active compounds or special fractions to optimize the efficacy of the extract. Nonetheless, this study provides evidence of the ethnopharmacological use of *C. racemosum*.

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