

Full Length Research Paper

***Mitragyna ciliata* and its trypanocidal activity**

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The trypanocidal activity of different fractions of hydroethanolic root extract of *Mitragyna ciliata* Aubrev and Pellegr (Rubiaceae) were evaluated in rats infected with *Trypanosoma brucei* field isolates from a cow. Oral administration of the fractions at a dose of 100 mg/kg for 5 days (10 days post-infection) indicated that only butanol fraction showed trypanocidal activity with inhibition percent of 68.68. The activities of oxidative stress enzymes; superoxide dismutase (SOD) and catalase in the infected rats were determined. SOD activity was significantly higher than control (1.64 ± 0.026 I/U) in all fractions except ethyl acetate (1.56 ± 0.031 I/U). Catalase showed a significant decrease in activity in butanol (2.05 ± 0.015 I/U) and chloroform (2.18 ± 0.061 I/U) fractions compared to control (2.30 ± 0.015 I/U). Butanol fraction might have affected the redox equilibrium of the infected animals causing oxidative stress to the parasites. This is the basis of inhibition of growth of the parasites by the butanol fraction.

Key words: *Mitragyna ciliata*, *Trypanosoma brucei*, oxidative stress, catalase and superoxide dismutase.

INTRODUCTION

Trypanosomiasis has recently become resurgent in Africa (WHO, 1998). This disease, restricted to Africa afflicts both human and livestock and causes great social and economic losses. World Health Organisation 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). Chemotherapy remains the principal means of control with existing trypanocides being faced with toxicity and drug resistance problems (FAO, 1998). Consequently, this scourge has become a pressing challenge especially to African medical scientists to find an alternative to synthetic drugs for the management of the disease. This has led to intensive research into African medicinal plants and many of these plants have been found to have antitrypanosomal activity (Atawodi et al., 2003; Freiburghaus et al., 1997; Nok et al., 1993).

Mitragyna ciliata stem bark extract mixed with *Garcinia*

kola is used for the treatment of trypanosomiasis in traditional medicine in South Eastern Nigeria (Iwu, 1993). The genus *Mitragyna* has been reported to be rich in alkaloids and many have been isolated from the leaves, stem and root bark. Isolated alkaloids include mitraphylline, rotundifoline, rhynchophylline and speciociliatine (Dongmo et al., 2004). The alkaloids from *Mitragyna inermis* have been shown to possess *in vitro* antiproliferative activity against *Plasmodium falciparum* (Traore et al., 2000). We had earlier reported that the ethanolic root bark extract of this plant possess trypanocidal activity (Ogbunugafor et al., 2005). In this present study, the effect of the fractions of hydroethanolic extract of *M. ciliata* on parasitaemia levels and on oxidative stress enzymes were evaluated in rats infected with *T. brucei* field isolates.

MATERIALS AND METHODS

Chemicals and solvents

All chemicals and solvents used were of analytical grade and were supplied by Sigma-Aldrich Limited Germany. The reference drug

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Somorenil – diminazine diacetate was purchased from Damax Agrochemical Company, Agege, Lagos.

Plant preparation

The fresh root of the plant was collected and authenticated at the Forestry Research Institute (FRIN) Ibadan, Nigeria, by Mr. Felix Isang and Mr. T. K. Odewo, respectively. Plant specimen with No. FHI:106999 *M. ciliata* Aubrey Pellerg (*Rubiaceae* local name Abura) was deposited at the FRIN herbarium.

Extraction and fractionation of plant material

Three hundred grams of air-dried powdered roots was extracted with ethanol/H₂O (8:2) by soaking for 72 h (3 days) at room temperature. This process was repeated several times to remove the extractable components. Combined extracts was evaporated to dryness under reduced pressure below 40°C (yield 20.34 g). The extract was fractionated by the method reported by Wu et al. (2005). The ethanolic extract was dissolved in 200 ml of MeOH:H₂O (9:1) mixture and shaken with petroleum ether (3 x 100 ml). Combined petroleum ether extract was evaporated under reduced pressure to yield 'Pet ether fraction' (4.16 g). MeOH was evaporated from the remaining extract and diluted with distilled H₂O to 200 ml and further fractionated by successive solvent extraction with chloroform (4 x 100 ml), ethyl acetate (2 x 100 ml) and n-butanol saturated with H₂O (3 x 100 ml). Each extract was evaporated to dryness under reduced pressure to yield 'CHCl₃ fr' (5.59 g) 'EtOAc fr' (6.90 g), 'BuOH fr' (1.46 g) and 'remaining H₂O fr' (2.0 g).

Parasites and animals

Parasites *T. brucei* were obtained from field isolates at the Department of Veterinary Parasitology and Entomology, University of Nigeria Nsukka. They were maintained by continuous passaging in the laboratory until needed. Thirty-five Wistar rats of mixed sexes, weighing between 200 – 250 g purchased from the animal house of College of Medicine, University of Lagos, were used for the experiment. The rats were sorted and grouped into 7 groups of 5 rats each and were acclimatised for 2 weeks.

In vivo antitrypanosomal evaluation of fractions

The grouped rats were inoculated with 10⁶ trypanosomes per ml of blood intraperitoneally using phosphate buffer saline as diluent. Two groups served as positive (reference drug) and negative (untreated) controls. The positive control group were administered with Somorenil – diminazine diacetate. Five groups served as the test groups and were administered with petroleum ether, chloroform, ethyl acetate, n-butanol, and water fractions. The fractions were dissolved in 5% carboxy methyl cellulose (CMC) serving as dosing vehicle.

The fractions were administered orally with the aid of oral cannular, to the infected rats at a dose of 100 mg/kg for 5 days. Reference drug was administered at 3.5 mg/kg also for five days. Parasitaemia levels were monitored for 28 days or until death of the rats and were estimated by Rapid Matching Method of Herbert and Lumsden (1976). Parasitaemia levels were compared with untreated control animals and percent inhibition of parasites assessed by the formula $100 - [\text{parasitaemia of test} / \text{parasitaemia of untreated} \times 100]$. Assessment of the fractions was also based on survival periods of the animals.

Free radical scavenging activity

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical by *M. ciliata* extract was determined according to the method reported by Gyamfi et al. (1999). *M. ciliata* ethanolic extract in methanol (50 µl), yielding a series of extract concentrations of 0.02, 0.04, 0.08 and 0.1 µg/ml, respectively were prepared. Each reaction was mixed with 100 µL of 0.1 mM DPPH-ethanol solution and 450 µL of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 µL) alone was used as control of this experiment. After 30 min of incubation at room temperature the reduction of the DPPH free radical was measured by reading the absorbance at 517 nm.

Vitamin E was used as positive control. The inhibition ratio (percent) was calculated from the following equation: %inhibition = [(absorbance of control- absorbance of test sample)/absorbance of control] x 100.

Serum collection

Tail blood (2 ml) was collected from the rats after administration of fractions and serum separated by centrifugation. Enzyme assay was carried out immediately and was done in triplicate.

Determination of catalase

Serum catalase activity was determined according to Beers and Sizer (1952) by measuring the decrease in absorbance at 240 nm in a UV recording spectrophotometer by monitoring the decomposition of H₂O₂ as described by Aebi (1984). The reaction mixture (3 ml vol) contained 0.1 ml of serum in phosphate buffer (50 mM, pH 7.0) and 2.9 ml of 30 mM H₂O₂ in phosphate buffer pH 7.0. An extinction coefficient for H₂O₂ at 240 nm of 40.0 M⁻¹cm⁻¹ Aebi (1984) was used for the calculation. The specific activity of catalase is expressed as moles of H₂O₂ reduced per minute per mg protein.

Determination of superoxide dismutase (SOD)

Whole blood superoxide dismutase was assayed utilizing the technique of Fridovich (1989). 1 ml of whole blood was diluted in 9 ml of distilled water to make a one in ten dilution of whole blood. An aliquot of 2.0 ml of the diluted blood was added to 2.5 ml of 0.05 M carbonate buffer (pH 10.2) and left to equilibrate in the spectrophotometer. The reaction was started by the addition of 0.3 ml freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3 ml of substrate (adrenaline) and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 s for 150 s. A single unit of enzyme is defined as moles of SOD required to produce 50% inhibition of autoxidation.

RESULTS AND DISCUSSION

The activity-guided fractionation of hydroethanolic root extract of *M. ciliata* revealed the butanol fraction as possessing the trypanocidal activity credited to this plant (Ogbunugafor et al., 2005) (Table 2). This fraction inhibited the growth of *T. brucei* field isolates by 68.68% at the end of 5 days oral administration. The inhibition was quite comparable to diminazine diacetate (the reference drug) which produced complete inhibition (Table 3).

Table 1. Scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical by *M. ciliata* extract.

Concentration ($\mu\text{g/ mL}$)	Percent inhibition	
	Control (Vitamin E)	<i>Mitragyna ciliata</i> *
00.02	62.20 \pm 0.624	06.96 \pm 0.054
0.04	88.93 \pm 0.423	14.71 \pm 0.046
0.08	89.93 \pm 0.552	53.20 \pm 0.227
0.10	90.29 \pm 0.320	34.21 \pm 0.034

*significantly lower ($p > 0.05$).

Table 2. Parasitemia levels in infected rats treated with fractions of *Mitragyna ciliata*.

Days	Untreated (-ve Control)	Diaceturate (+ve Control)	Butanol	EtOAc	CHCl ₃	Pet Ether	Water
0*	7.01 \pm 0.15	8.21 \pm 0.72	6.28 \pm 0.35	5.09 \pm 0.41	16.44 \pm 4.2	8.48 \pm 2.4	7.88 \pm 2.10
3#	3.15 \pm 0.04	1.80 \pm 0.02	0.79 \pm 0.04	6.59 \pm 0.25	1.25 \pm 0.02	0.27 \pm 0.03	3.54 \pm 0.25
5#	2.21 \pm 0.24	0.26 \pm 0.01	0.86 \pm 0.02	0.63 \pm 0.02	3.36 \pm 0.23	4.83 \pm 0.15	6.01 \pm 2.42
7#	8.00 \pm 1.22	0.00 ⁺	2.49 \pm 0.35 ⁺	6.10 \pm 0.32	20.04 \pm 8.5	20.30 \pm 9.0	10.45 \pm 3.8

Values are $\times 10^6/\text{ml}$ of blood.

*Before administration of extracts.

#After administration of extracts.

*significantly lower ($p > 0.05$).

Table 3. Percent inhibition of fractions of *Mitragyna ciliata*.

DAYS	Diaceturate (+ve Control)	Butanol	EtOAc	CHCl ₃	Pet Ether	Water
3	42.86	74.82	-109.20	60.65	91.40	-12.83
5	88.23	53.33	71.38	-52.51	-118.59	-172.56
7	100.00	68.68	23.75	-150.52	-153.75	-30.63
Survival # period (days)	>28	>28	8	5	3	8

#After administration of extract.

Table 4. Activities of SOD and catalase in infected rats after administration of fractions of *Mitragyna ciliata*.

Enzyme	Untreated	Butanol	EtOAc	CHCl ₃	Pet Ether	Water
SOD (I/U)	1.64 \pm 0.026	1.73 \pm 0.051*	1.56 \pm 0.03	2.29 \pm 0.04*	2.62 \pm 0.026*	1.85 \pm 0.02*
Catalase (I/U)	2.30 \pm 0.015	2.05 \pm 0.051*	2.33 \pm 0.025	2.18 \pm 0.061*	2.30 \pm 0.04	2.34 \pm 0.015

*significant ($p > 0.05$).

The effect of the plant extract on the infected animals was evaluated based on the non concentration dependent antioxidative activity of the crude extract (Table 1). The antioxidative activity was significantly lower than control (Vitamin E). Thus, the oxidative stress enzymes - superoxide dismutase (SOD) and catalase, of the infected rats were assayed, after oral administration of the fractions. Results showed significantly elevated levels of superoxide dismutase in all fractions, except ethyl acetate (Table 4), indicating an increase in superoxide

free radicals in the infected rats (Usoh et al., 2005). The physiological functions of catalase and SOD are well known (Yoruk et al., 2005) we infer that the creation of oxidative stress in the animals will affect the parasites. This is buttressed further in the butanol fraction treated rats which coupled elevated ($p > 0.05$) SOD activity to significantly depressed ($p > 0.05$) activity of catalase (Table 4). Hydrogen peroxide is produced from the dismutation of free oxygen radicals by SOD, even though the natural substrate of catalase acts also as its own inhi-

bitor (Aksoy et al., 2004). Subsequently, the accumulated H_2O_2 alters the redox equilibrium of the animals (increasing oxidative stress) in the butanol fraction treated rats. This seems not to be injurious to the rats but to the parasites resulting in their inhibition. Moreover, superoxide anion inhibits catalase converting it to the ferroxyl and ferryl inactive forms of the enzyme (Kono and Fridiwich, 1982). SOD has also been shown to be inactivated by H_2O_2 (Rister and Bachner, 1976).

Furthermore, the host (rats) and the trypanosomes have in common superoxide dismutases in their enzymatic defence systems against free radicals (Soulere et al., 1999) but the parasite lacks catalase (Wang et al., 1999). In the absence of catalase the parasite depends solely on a spermidine-gluthathione conjugate named trypanothione whose oxidative form is regenerated by trypanothione reductase that is very sensitive to alteration in redox equilibrium (Atawodi et al., 2003). Therefore, the depression in catalase activity in the infected rats, which arose from its inhibition by accumulated H_2O_2 , might have caused peroxidative damage to trypanothione reductase. This damage caused disruption in the defence of the parasites against oxidative stress. Consequently, the parasites became vulnerable and their membranes were undermined. The observation that depression in catalase activity coincides with clearance or inhibition of parasites' proliferation is supported by earlier work that *T. brucei* clearance from the blood of Cape buffalo coincided with the time catalase activity was depressed (Wang et al., 1999). In addition, parasitic protozoan are known to be susceptible to free oxygen radical-induced oxidative stress (Clark and Hunt, 1983). Also, our results are consistent with earlier reports that natural products affect their trypanocidal activity by creating oxidative stress (Sepulveda-Boza et al., 1996).

The observation that the activity of SOD was not elevated in the ethyl acetate fraction is consistent with previous studies. Ethyl acetate fraction from various plant extracts is known for its antioxidative property which is attributable to the extractable phenolic compounds in this fraction (Wu et al., 2005).

This report suggests the basis of the trypanocidal activity of the ethanolic root extract of *M. ciliata* and corroborates its use by traditional healers in South Eastern Nigeria for the treatment of trypanosomiasis

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