

*Full Length Research Paper*

## ***In vitro* anti-trypanosomal activity of *Morinda lucida* leaves**

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**Bioassay-guided fractionation of the crude methanol extract of *Morinda lucida* Benth. (Rubiaceae) was carried out by column chromatography and anti-trypanosomal activity was tested using *in vitro* cultures of *Trypanosoma brucei brucei* S427. Three active fractions (ML 13-18, ML 19-28 and ML 33-45) were obtained with minimum inhibitory concentration (MIC) values of 6.25, 25 and 12.5 µg/ml, respectively. Fraction ML 33-45 was identified as β-sitosterol/stigmasterol using nuclear magnetic resonance (NMR). β-Sitosterol was evaluated for its cytotoxicity using normal prostate cells (PNT2A) and prostatic carcinoma cell lines (DU-145, PC-3 and LNCAP AS) at a concentration of 100 µM. β-Sitosterol was cytotoxic to all the cell lines.**

**Key words:** *Morinda lucida*, *Trypanosoma brucei brucei*, beta sitosterol, stigmasterol, cytotoxicity.

### **INTRODUCTION**

Trypanosomes are flagellated haemoparasites that are widely distributed in the animal kingdom (Atawodi et al., 2002). They are the causative agents of serious debilitating diseases of man and animals in vast areas of sub-Saharan Africa where the disease is endemic. Human infections with trypanosomes are called human african trypanosomosis (HAT), while animal infections are known as african animal trypanosomosis (AAT). These infections are more prevalent in the rural areas (Atougua and Costa, 1999) and are transmitted through the bite of infected tsetse flies (*Glossina* spp.). Trypanosomosis is of great significance to human health and animal production in Africa (Bizimana et al., 2006).

In the absence of effective vaccination, control of trypanosomosis is principally achieved by means of either chemoprophylactic or chemotherapeutic agents (Maser et al., 2003; Antia et al., 2009). Most of these drugs for the control of both animal and human trypanosomoses are chemically related (Bizimana et al., 2006) and have been in use for decades. Hence, the repeated use of these trypanocides has led to the development of drug-resistant trypanosome populations. Also, most of these available trypanocides are bedevilled by many undesirable toxic

side effects (Deterding et al., 2005) that are serious and even life-threatening (Hoet et al., 2007). There is need for new, effective, safe, easy-to-administer and inexpensive trypanocides.

Nature with its numerous organisms is a potential source of such new drugs since it contains countless quantities of molecules with great varieties of structures and pharmacological activities (Newman et al., 2003). The use of medicinal plants has a long history, especially, in Africa. In fact, herbs have been utilized by people throughout history. It is estimated that two-third of the world population rely on traditional medical remedies due to the limited availability and affordability of pharmaceutical products (Tagboto and Townson, 2001).

*Morinda lucida* Benth. (Rubiaceae) is a medicinal plant. The Igbo of South Eastern Nigeria call it '*nfia*'. The leaves are very bitter and are used by the natives to treat malaria, induce purgation, emesis and diuresis (Anaga, 2003). Three compounds (digitolutein, rubiadin 1-methyl ether and damnacanthol) isolated from the stem bark and roots of *M. lucida* significantly decrease the number of *Plasmodium falciparum* schizonts *in vitro* (Koumaglo et al., 1992). A 100% inhibition was achieved with 30 to 40

mg of each compound tested. The crude methanol extract of *M. lucida* leaves induced purgation in mice and suppressed the level of parasitaemia in *Trypanosoma brucei brucei* infected mice in a dose-dependent manner (Asuzu and Chineme, 1990). Suppression of parasitaemia appeared dose-dependent with 1000 mg/kg i.p. producing the maximum effect. These workers reported that the best trypanocidal activity was obtained when treatment with *M. lucida* extract commenced simultaneously with trypanosome inoculation.

Other activities reported from the crude methanol extract of *M. lucida* and its fractions include anti-inflammatory, antipyretic and analgesic activities in rats (Awe et al., 1998). The crude methanol extract also potentiated pentobarbitone-induced sleeping time of rats. Other workers reported reversible antispermatogenic effects of crude *M. lucida* extract on male rats treated for 14 to 28 days (Raji et al., 2005).

The aim of this work was to identify through bioassay-guided fractionation the active compounds responsible for the observed anti-trypanosomal activity of *M. lucida* leaves and to evaluate the cytotoxicity.

## MATERIALS AND METHODS

*M. lucida* leaves were collected from Nsukka and identified by Mr A. O. Ozioko of the Botany Department, University of Nigeria, Nsukka. The leaves were dried and pulverised. Extraction was done using 50% methanol and 50% distilled water. The extract was stored at -20°C before use.

### Phytochemical screening of extract and fractions

Thin layer chromatography (TLC) of the plant extract was carried out by using pre-coated silica gel plates (0.063 to 0.020 mm, Kieselgel 60 PF<sup>254</sup>, Merck No 5554). The mobile phase was 10% ethyl acetate in hexane. Following development and drying, chromatograms were examined under short (254 nm) and long (366 nm) UV light. Spots on TLC were visualized using anisaldehyde-sulphuric acid spray. Column chromatography of the 50% methanol extract was done using column grade silica gel (silica gel 60, Merck) in a glass column and eluted gradient-wise with ethyl acetate in hexane. Eluates collected were monitored by TLC and bulked as appropriate.

### Structural elucidation

Characterisation of ML 33-45 was achieved by nuclear magnetic resonance (NMR). The <sup>1</sup>H NMR spectra were run in a JEOL Eclipse (400 MHz), while the <sup>13</sup>C NMR spectra were run on a Bruker spectrophotometer (600 MHz). Deuterated dimethylsulphuroxide (DMSO) was used as solvent.

### *In vitro* determination of antitrypanosomal activity

An Alamar blue<sup>®</sup> assay was used to determine the *in vitro* anti-

trypanosomal activity of crude *M. lucida* leaves extract and fractions. This was done according to the method of Raz et al. (1997). The samples were prepared as stock solutions in DMSO at a concentration of 10 mg/ml. The concentration of *T. brucei brucei* S427 was 2 to 3 × 10<sup>4</sup> trypanosomes/ml.

### Initial screening with one concentration

A tenfold dilution was made by adding 5 µl of the extracts and fractions to 45 µl of HMI-9 media in a round-bottomed 96 well microtitre plate. There were also four control wells (1E to H) with 5 µl of DMSO and 45 µl of HMI-9 media. This dilution plate was placed on a microtitre shaker for about 1 min in order to thoroughly mix the contents. 4 µl of the contents of the dilution plate were transferred to the corresponding wells of a flat bottom 96 well microtitre plate. HMI-9 media (96 µl) was added to all wells up to 100 µl each, except column 12 wells which received 80 µl of the media only. Column 12 wells served as positive control wells which received 20 µl of Suramin. 100 µl of the blood stream form of trypanosomes were added to each well and incubated at 37°C, and 5% CO<sub>2</sub> in a humidified atmosphere for 48 h. After incubation for 48 h, 20 µl of Alamar blue (REDOX indicator, Abdsertec, UK) was added. The assay plate was incubated under conditions previously described for further 24 h. Fluorescence was determined by using the Wallac Victor apparatus at an excitation wavelength of 530 nm and emission wavelength of 590 nm.

### Determination of minimum inhibitory concentration (MIC)

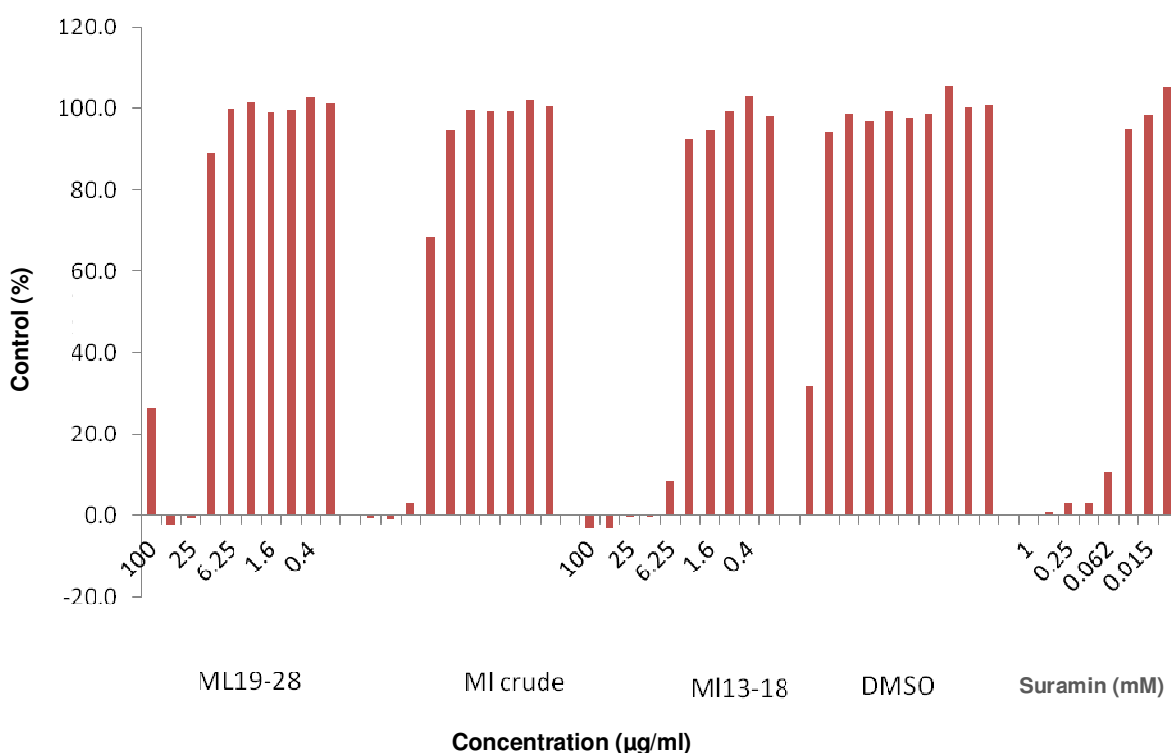
Briefly, 4 µl of the test samples were added in duplicate to column 2 of a microtitre plate. HMI-9 medium (196 µl) was added to the same wells to a final volume of 200 µl. All other wells received 100 µl each, except those on column 12 which received 80 µl. Serial double dilutions were carried out from column 2 to 11. A solution of Suramin (Sigma, UK) was prepared and filter sterilized to give a final concentration range of 1 to 0.008 µM. 20 µl of each concentration was added to the corresponding well of column 12. 100 µl of a suspension of the blood stream form of *T. b. brucei* S 427 was added to each well. The assay plate was incubated and the results were read as described earlier.

### Determination of cytotoxicity

The cytotoxicity of *M. lucida* fraction 33-45 was studied at a concentration of 100 µM using normal prostate cells (PNT2A) and prostatic carcinoma cell lines (DU-145, PC-3 and LNCAP AS). Dulbecco's modified eagles medium (DMEM) was used for all the cells. Triton X was used at a final concentration of 0.1%, that is, 10 µl in 2.5 ml DMEM (400 µl / plate). On the first day, 75 µl of cells were seeded at appropriate concentrations (DU-145 at 0.5 × 10<sup>5</sup>; LNCAP AS at 10<sup>5</sup>; PC-3 at 10<sup>5</sup> and PNT2A at 0.5 × 10<sup>5</sup>) and in appropriate cell medium. The assay plates were incubated overnight at 37°C in the presence of 5% CO<sub>2</sub> and 95% O<sub>2</sub>. On the second day, 25 µl of the samples (which are 4 x final concentration) were added to the wells of A2-H11. 25 µl of the medium was added to Column 1 and 25 µl of Triton X was added to column 12. For 24 h sample incubation, 10 µl of Alamar Blue was added to all the wells. The plates were incubated for 24 h at 37°C in the presence of 5% CO<sub>2</sub>, 95% O<sub>2</sub> and read on Wallac Victor in fluorescence mode (AB560/590 nm). For 48 h sample incubation, 10 µl of Alamar Blue

**Table 1.** Phytochemical properties of *M. lucida* fractions.

Fraction	TLC	Yield (mg)	UV (short)	Rf	Remark / NMR
ML1-3	1 spot	92.7	-	0.76	Chlorophyll
ML4-12	3 spots	72.8	2 spots	-	-
ML13-18	3 spots	51.7	1 spot	-	Long chain alcohol
ML 19-28	4 spots	77.3	1 spot	-	fatty acid
ML 29-32	3 spots	38.1	1 spot	-	-
ML 33-45	1 spot	130	1 spot	0.1	$\beta$ -Sitosterol/stigmasterol
ML 46-49	1 spot	32.6	1 spot	0.06	aromatic/terpenoid compound
ML 50-58	2 spots	46.6	1 spot	0.04	-
ML 59-72	1 spot	89	1 spot	0.03	-

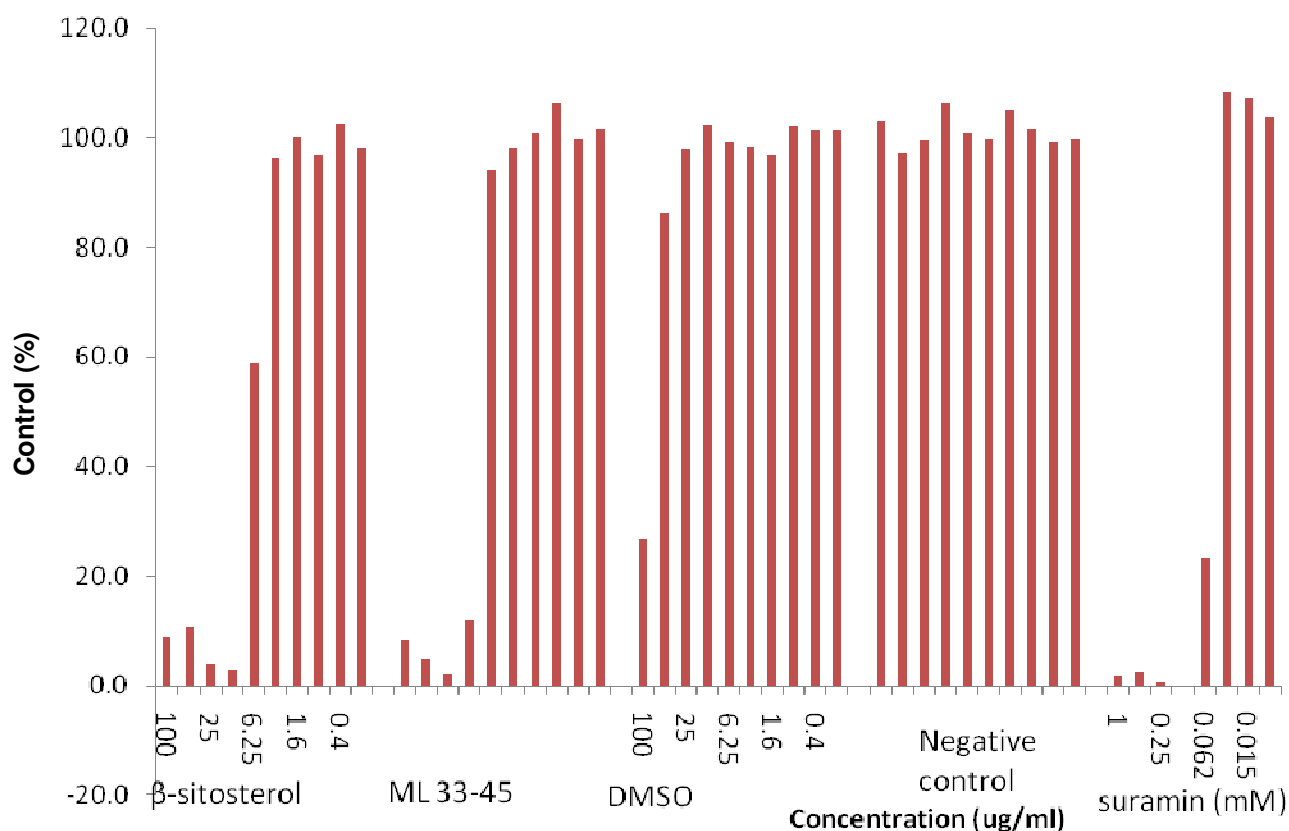
**Figure 1.** Minimum inhibitory concentrations of *M. lucida* fractions tested on *T. brucei brucei* S427.

was added to all the wells after 48 h and the plates were read the following day.

## RESULTS

The result of the phytochemical screening of *M. lucida* crude extract and fractions is shown in Table 1. The

result of the anti-trypanosomal screening is shown in Figures 1 and 2. *Morinda lucida* crude extract, fractions 13-18, 19-28 and 33-45 showed anti-trypanosomal activities with MIC values of 25, 6.25, 25 and 12.5 µg/ml, respectively. Fraction 33-45 was identified as a combination of  $\beta$ -sitosterol and stigmasterol by NMR. The NMR spectra of the other active fractions could not be conclusively determined. The result of the cytotoxicity



**Figure 2.** Minimum inhibitory concentrations of *M. lucida* fractions and  $\beta$ -sitosterol on *T. b. brucei*.

study of  $\beta$ -sitosterol on the different cell lines is shown in Figure 3.  $\beta$ -Sitosterol had cytotoxic effects on all the cell lines with no evidence of selectivity. Its percentage of control at a concentration of 100  $\mu$ M was 136, 140, 135 and 145 against DU-145, LNCaP AS, PC-3 and PNT2A cell lines, respectively.

## DISCUSSION

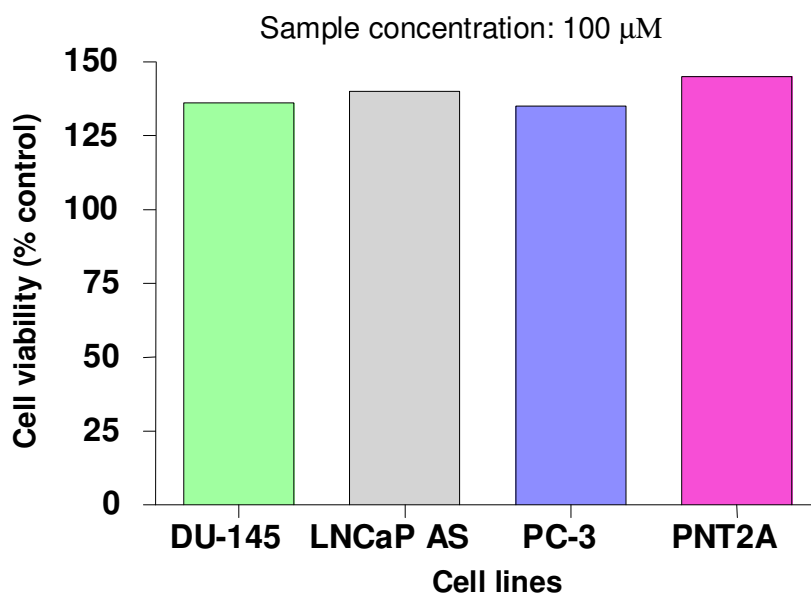
The crude methanol extract of *M. lucida* showed anti-trypanosomal activity against *T. b. brucei* S427 bloodstream forms *in vitro* with an MIC value of 25  $\mu$ g/ml. This is a confirmation of the earlier report of its anti-trypanosomal activity by Asuzu and Chineme (1990). It also corroborates its ethnomedical use as a medicinal plant. Of the three active fractions obtained by column chromatography, fraction 13-18 showed the highest activity with an MIC value of 6.25  $\mu$ g/ml. Fractions 33-45 and 19-8 had MIC values of 12.5 and 25  $\mu$ g/ml, respectively.

Fraction 33-45 was shown to be a mixture of  $\beta$ -

Sitosterol and stigmasterol by NMR. The structures of fractions 13-18 and 19-28 could not be conclusively determined. Cytotoxicity testing of  $\beta$ -sitosterol using both normal and cancerous prostatic cell lines showed evidence of cytotoxic activity with no selectivity because the concentration was high and with only few cell lines. This lack of selectivity is a severe drawback to its use.

Other sterols that have been reported to have anti-trypanosomal activities are vernoguinsterol and vernoguinoside isolated from the stem bark of *Vernonia guineensis* (Tchinda et al., 2002). They were shown to have significant inhibitory activity against the bloodstream trypomastigote forms of *T. brucei rhodesiense* with  $IC_{50}$  values between 3 and 5  $\mu$ g/ml. Also, fractionation of the lipophilic extracts of the leaves of *Strychnos spinosa* yielded two sterols namely, saringosterol and 24-hydroperoxy-24-vinylcholesterol which were found to possess *in vitro* anti-trypanosomal activities against *T. b. brucei* (Hoet et al., 2007). Their  $IC_{50}$  values were  $7.8 \pm 1.2$  and  $3.2 \pm 1.2$   $\mu$ M, respectively.

$\beta$ -sitosterol has been shown to possess anticancer activities. It was found to inhibit breast and prostate



**Figure 3.** The cytotoxic effect of  $\beta$ -sitosterol on DU-145, LNCaP AS, PC-3 and PNT2A cell lines.

cancer cell growth as well as been able to induce cell cycle arrest (Strum and Faloon, 2008). There are claims that men affected by benign prostate tumour use  $\beta$ -sitosterol either alone or in combination with other drugs for treatment (Gerber, 2002). The *in vitro* cytotoxicity for various cancer cell lines of the sterol peroxide 24-hydroperoxy-24-vinylcholesterol has also been documented (Ktari and Guyot, 1999).

Interestingly, another plant shown to have anti-trypanosomal activity is *Annona senegalensis* Pers., which was able to clear *T. brucei* infection in mice within 3 days of consecutive treatment with the crude water extract of the leaves (Ogbadoyi et al., 2007). In this report, it was noted that the same plant is used by the natives in the treatment of cancer (Gbile and Adesina, 1985). Also, according to Barrett and Barrett (2000), all four drugs currently used in the treatment of clinical trypanosomiasis have anti-cancer properties because they are cytotoxic. This includes di-fluoromethylornithine (DFMO) which was originally developed as an anti-cancer drug. In another study, 15 DNA topoisomerase inhibitors (a class of anticancer drugs), were found to possess *in vitro* anti trypanosomal activities against *T. brucei* which is comparable to that of the commercial anti-trypanosomal drug, pentamidine (Deterding et al., 2005). Similar finding of anti-trypanosomal activity was reported on camptothecin, which belongs to the same class of DNA topoisomerase inhibitors (Bodley and Shapiro, 1995). Even suramin, which is used in treating the early

stage of HAT, has been shown to be a topoisomerase II inhibitor in mammalian cells (Bojanowski et al., 1992). Likewise, melasorprol (which is used in the treatment of late-stage trypanosomiasis) was shown to induce apoptosis in cancer cell lines (Konig et al., 1997).

The crude methanol extract of the leaves of *M. lucida* and fractions 13-18, 19-28 and 33-45 have been shown to possess anti-trypanosomal activities against *T. b. brucei* S427 blood stream forms *in vitro* with MIC values of 25, 6.25, 25 and 12.5  $\mu$ g/ml, respectively. Fraction 33-45 was identified to be a mixture of  $\beta$ -sitosterol and stigmasterol. Further work is needed to identify the other active fractions.

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