

Full Length Research Paper

## In vitro antileishmanial activity of extracts of *Warburgia ugandensis* (Canellaceae), a Kenyan medicinal plant

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The antileishmanial activity of extracts of *Warburgia ugandensis* (family Canellaceae), a known traditional therapy and one of the commonly used medicinal plants in Kenya was evaluated. Extracts of this plant were tested for possible antileishmanial activity in vitro. Different doses of hexane, dichloromethane, ethyl acetate and methanol extracts of *W. ugandensis* were tested against *Leishmania major* and *Leishmania donovani* promastigotes and amastigotes. The hexane extract had the best activity against *L. major* promastigotes and amastigotes with  $IC_{50}$  value of 9.95 for promastigotes and 8.65 for amastigotes and minimum inhibition concentrations of 62.5  $\mu$ g/ml. The activity of the hexane extract on amastigotes was comparable ( $P > 0.05$ ) to that of pentostam and amphotericin B. Similar results were obtained for *L. donovani* with  $IC_{50}$  values of 8.67 for promastigotes and 100-fold reduction of amastigotes in macrophage cultures. *Warburgia ugandensis* had lower levels of toxicities compared to pentostam and amphotericin B. This study scientifically demonstrates the potential of *W. ugandensis* in the treatment of leishmaniasis.

**Key words:** *Warburgia ugandensis*, leishmaniasis, promastigote, amastigote.

### INTRODUCTION

The leishmaniasis are diseases caused by obligate intracellular, kinetoplastid protozoa of the genus *Leishmania* (Trypanosomatidae) (Roberts, 2006). Although it is not a household name like malaria, the diseases caused by infection with *Leishmania* continue to have a major impact on much of the world's population and are currently considered to be an emerging illness with high morbidity and mortality in the tropics and subtropics (Handman, 2001; Santos et al., 2008).

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### Abbreviations

**FBS**, Foetal bovine serum; **KEMRI**, Kenya Medical Research Institute; **ACUC**, Animal Care and Use Committee; **MTT**, Thiazolyl blue tetrazolium bromide; **VL**, Visceral leishmaniasis; **ANOVA**, Analysis of variance.

Neglected by researchers and funding agencies, leishmaniasis are endemic in 88 countries of the world in which 350 million people who are considered at risk of infection live (WHO, 2007). There are 2 million new cases of leishmaniasis annually and 14 million infected people worldwide (WHO, 2007).

An increase in the incidence of leishmaniasis can be associated with urban development, destruction of forests, environmental changes, migrations of people to areas where the disease is endemic and wars which contribute to its spread due to displacement of people (Ashford, 2000; Patz et al., 2000; Kolaczinski et al., 2007).

Proven therapies against human leishmaniasis include pentavalent antimonials (sodium stibogluconate and meglumine antimoniate), amphotericin B, pentamidine, miltefosine and paromomycin (Berman; 1996, 1997). These drugs are unsatisfactory because of their limited efficacy, frequent side effects and increasing drug resistance,

therefore, new, safer and more efficacious drugs are urgently required (Croft et al., 2005). Moreover, there is no effective vaccine against leishmaniasis (Handman, 2001). In this regard, medicinal plants offer prospects for discovering new compounds with therapeutic properties.

*Warburgia ugandensis* Sprague, the East African green-heart (Canellaceae) is one of the most highly utilized medicinal plants in tropical and subtropical Africa and is now highly endangered in the wild (Kioko et al., 2005). It is rated as the second highest priority medicinal plant species in Kenya (Kariuki and Simiyu, 2005). According to the World Agroforestry Centre, (<http://www.worldagroforestrycentre.org/sea/products/AFDBases/af/asp/SpeciesInfo>), the dried bark of the tree is commonly chewed and the juice swallowed as a remedy for stomach ache, constipation, toothache, venereal diseases, cough, fever, muscle pains, weak joints and general body pains. The leaf decoction baths are used as a cure for skin diseases while the bark, roots or leaves can be boiled in water and drunk to treat malaria, although this causes violent vomiting.

*Warburgia ugandensis* which is known as "soket" in Tugen tribe is used by traditional healers to treat visceral leishmaniasis (VL) (W. Tonui, Kenya Medical Research Institute, Nairobi, personal communication). The stem barks are taken orally in boiled water or soup.

Previous studies on *W. ugandensis* have shown good antibacterial, antifungal, antiviral activity and trypanocidal effects. Crude extracts and purified compounds of *W. ugandensis* showed activity against *Mycobacterium tuberculosis* H37Rv and *M. Bovis* BCG Pasteur (Madikane et al., 2007), *Candida albicans* and measles virus (Olila et al., 2001;2002). Similarly, the activity of *W. ugandensis* against trypanosomes has been demonstrated (Kioy et al., 1998; Olila et al., 2001). However, the effect of the plant on *Leishmania* parasites has not been documented.

The objective of the present study was to determine the effect of extracts of *W. ugandensis* in vitro on the growth and viability of *L. major* and *L. donovani* promastigotes in cell-free culture and amastigotes in murine macrophages.

## MATERIALS AND METHODS

### Source of *W. ugandensis*

*W. ugandensis* stem barks were collected from Sesia village in Kabarnet, Baringo District, in the Rift Valley Province in Kenya. Botanical identification was done using standard identification keys by the National Museums of Kenya botanists. Voucher specimens were kept in the Kenya Medical Research Institute laboratory in Nairobi.

### Sample preparation and extraction of compounds of *W. ugandensis*

The stem barks were cut into small pieces and air-dried for three weeks under a shed. The dried specimens were shred using an electrical mill in readiness for extraction. The sample preparation

and extraction procedure were carried out as described by Harbone (1994). Cold sequential extraction was carried out on plant material with analar grade organic solvents of increasing polarity, which included n-hexane, dichloromethane, ethyl acetate and methanol. Six hundred millilitres of n-hexane was added to 300 g of the shred specimen and flasks placed on a shaker and soaked for 48 h. The residue was filtered using a Buchner funnel under vacuum until the sample dried. The sample was soaked further with 600 ml of n-hexane for 24 h until the filtrate remained clear. The filtrate was then concentrated under vacuum by rotary evaporation at 30 - 35°C (Harborne, 1994). The concentrate was transferred to a sample bottle and dried under vacuum; the weight of the dry extract was recorded and stored at -20°C until required for bioassay. The process was repeated sequentially for dichloromethane, ethyl acetate and methanol.

### Bioassays for antileishmanial activity of *W. ugandensis*

**Experimental animals:** Eight week old BALB/c mice for macrophage assays were obtained from Kenya Medical Research Institute's (KEMRI) animal house facility. The experiments using mice were done in compliance with Animal Care and Use Committee (ACUC) guidelines of KEMRI.

**Leishmania parasites:** Metacyclic promastigotes of *L. major* strain (Strain IDU/KE/83 =NLB-144) and *L. donovani* (strain NLB-065) were used. Parasites were maintained as previously described (Titus et al., 1984) and metacyclics were isolated from stationary phase cultures by negative selection using peanut agglutinin (Tonui et al., 2004). Briefly, promastigotes were cultured in Schneider's *Drosophila* medium supplemented with 20% foetal bovine serum (FBS), penicillin G (100 U/ml), and streptomycin (100 µg/ml). Stationary-phase promastigotes were obtained from 5 to 7 day-old cultures.

**Preparation of drugs:** Stock solutions of the fractions were made in culture media for anti-leishmanial assay and re-sterilized by passing through 0.22 µm micro-filters under sterile conditions in a laminar flow hood. The extracts that were insoluble in water or media were first dissolved in 1% DMSO to avoid solvent carry over, (Dorin et al., 2001). All the prepared drugs were stored at 4°C and retrieved only during use.

**Evaluation of minimum inhibitory concentration (MIC):** *Leishmania* promastigotes ( $10^6$  parasites/ml) were maintained in culture or in the presence of several concentrations (1mg/ml to 1µg/ml) of test compounds. Cell growth was evaluated daily by assessment of visibility turbidity in order to evaluate MIC. The lowest concentration of the samples that prevented the growth of *Leishmania* parasites *in vitro* was considered as the MIC.

**(i) Anti-promastigote assay:** *L. major* and *L. donovani* promastigotes were cultured in NNN media overlaid with 2 ml of Schneider's *Drosophila* insect medium (SIM-F) supplemented with 20% foetal bovine serum, 100 (g/ml streptomycin and 100 U/ml penicillin-G, and 5-fluorocytosine. Promastigotes were incubated in 24-well plates. After five days of cultivation, aliquots of parasites were transferred to 96-well micro-titer plate. The parasites were incubated at 27°C for 24 h and 200 µl of the highest concentration of each of the test samples was added and serial dilution carried out. The experimental plates were incubated further at 27°C for 48 h. The controls used were promastigotes with no drugs and medium alone (no drugs and no cells).

Ten microlitres of thiazolyl blue tetrazolium bromide (MTT) reagent was added into each well and the cells incubated for 2 - 4 h until a purple precipitate was clearly visible under a microscope.

The medium together with MTT was aspirated off from the wells, a hundred microlitres of DMSO added and the plates shaken for 5 min. Absorbance was measured for each well at 562 nm using a micro-titer plate reader (Mosmann, 1983) and the 50% inhibitory concentration ( $IC_{50}$ ) values generated. Percentage promastigote viability was calculated using the formula of Mosmann, 1983 at each concentration.

Promastigote viability (%) =  

$$\frac{\text{Average absorbance in duplicate drug wells} - \text{average blank wells}}{\text{Average absorbance control wells}} \times 100$$

Average absorbance control wells

**(ii) Anti-amastigote assay:** The anti-amastigote assay was carried out as described by Delorenzi *et al.* (2001). Briefly, peritoneal macrophages were obtained from two BALB/c mice. The body surface was disinfected, the skin torn dorso-ventrally to expose the peritoneum and a sterile syringe used to inject 10 ml of phosphate buffered saline (PBS) into the peritoneum. Mouse peritoneal macrophages were harvested by withdrawing the fluid into sterile centrifuge tubes. The cell suspension was centrifuged at 2000 rotations per minute at 40°C for 10 minutes and the pellet re-suspended in 5 ml of complete RPMI 1640 medium.

Macrophages were absorbed in 24-well plates and allowed to adhere for 4 hours at 37°C in 5% CO<sub>2</sub>. Non-adherent cells were washed with phosphate buffered saline (PBS), and the macrophages incubated overnight in RPMI 1640 media. Adherent macrophages were infected with *L. major* promastigotes at a parasite/macrophage ratio of 6:1, incubated at 37°C in 5% CO<sub>2</sub> for 4 h, free promastigotes removed by extensive washing with PBS, and the cultures incubated in RPMI 1640 medium for 24 h. Treatment of infected macrophages with the sample was done once. Pentostam® and amphotericin B were used as positive controls for parasite growth inhibition. The medium and drug was replenished daily for 3 days. After 5 days the monolayers were washed with PBS at 37°C, fixed in methanol and stained with 10% Giemsa solution. The number of amastigotes were determined by counting at least 100 macrophages in duplicate cultures, and results expressed as infection rate (IR) and multiplication index (MI) (Berman and Lee, 1984).

IR = No. of infected macrophages in 100 macrophages

MI =  

$$\frac{\text{No. of amastigotes in experimental culture}/100 \text{ macrophages}}{\text{No. of amastigotes in control culture}/100 \text{ macrophages}} \times 100$$

The infection rate was used in the calculation of the association index (AI). The AIs were determined by multiplying the percentage of infected macrophages by the number of parasites per infected cell. Association indices were the number of parasites that actually infected the macrophages.

#### Nitric oxide production determination

Nitric oxide release in supernatants of macrophage culture was measured by the Griess reaction for nitrites (Holzmuller *et al.*, 2002). Briefly, 100 µl of the supernatants were collected 48 hours after introducing the test drug into the culture medium. The assay was done in triplicate wells in a 96-well micro-titer plate. To this, 60 µl of Griess reagent A (1% sulphanilamide in 1.2 M HCl) was added, followed by 60 µl of Griess reagent B (0.3% N-[1-naphthyl] ethylenediamine). The plates were read at 540 nm in an ELISA plate reader. Sodium nitrite in RPMI was used to construct a standard curve for each plate reading.

#### Cytotoxicity assay

VERO cells were cultured and maintained in Minimum Essential

Medium supplemented with 10% FBS. The cells were cultured at 37°C in 5% CO<sub>2</sub>, harvested by trypsinization, pooled in a 50 ml vial and 100 µl cell suspension ( $1 \times 10^5$  cell/ml) put into 2 wells of rows A-H in a 96-well micro-titer plate for one sample. The cells were incubated at 37°C in 5% CO<sub>2</sub> for 24 h to attach, the medium aspirated off and 150 µl of the highest concentration of each of the test samples serially diluted. The experimental plates were incubated further at 37°C for 48 h. The controls used were cells with no drugs, and medium alone (no drugs and no cells). MTT reagent (10 µl) was added into each well and the cells incubated for 2 - 4 h until a purple precipitate was clearly visible under a microscope. The medium together with MTT was aspirated off from the wells, after which 100 µl of DMSO was added and the plates shaken for 5 minutes. The absorbance was measured for each well at 562 nm using a micro-titer plate reader (Wang *et al.*, 2006). Cell viability (%) was calculated at each concentration as described by Wang *et al.* (2006) using the formula:

Cell viability (%) =  

$$\frac{\text{Ave absorbance in duplicate drug wells} - \text{average blank wells}}{\text{Average absorbance control wells}} \times 100$$

#### Statistical analysis

All experiments were done in triplicate. The mean and standard deviation of at least three experiments were determined. The data was analyzed using SPSS 13.0 programme. Statistical analysis of the differences between mean values obtained for the experimental groups were done by analysis of variance (ANOVA) and student's t test. P values of 0.05 were considered significant.

## RESULTS

#### Extraction of compounds of *Warburgia ugandensis*:

Three hundred grams of the stem bark of *W. ugandensis* yielded 10.5 g of n-hexane extract, 12.1 g of dichloromethane, 9.3 g of ethyl acetate and 9.9 g of methanol extract. There was no significant difference ( $p > 0.05$ ) in the yields obtained.

#### Activity of extracts of *W. ugandensis* on *L. major* and *L. donovani* promastigotes:

The  $IC_{50}$  values for the hexane, dichloromethane and ethyl acetate extracts for *L. major* promastigotes were significantly different ( $p < 0.01$ ). Those for *L. donovani* were comparable and not statistically different ( $p > 0.05$ ). Methanol did not show activity against *L. major* and *L. donovani* promastigotes (Table 1). The  $IC_{50}$  and MIC values for hexane and dichloromethane samples were significantly lower ( $p < 0.001$ ) than those of pentostam but compared well with those of amphotericin B ( $p > 0.05$ ). Ethyl acetate had good activity ( $IC_{50}$  value below 100 µg/ml) against *L. donovani* promastigotes but did not work as well for *L. major* promastigotes ( $IC_{50}$  value above 100 µg/ml). The MIC values for *L. major* were lower ( $p = 0.02$ ) than those of *L. donovani* with those of *L. donovani* ranging from 250 to 500 µg/ml compared to those of *L. major* that ranged from 62.3 to 250 µg/ml (Table 1).

*Leishmania major* promastigotes treated with methanol extracts had the highest viability at all concentrations followed by dichloromethane. Promastigotes treated with

**Table 1.** *In vitro* activity of *Warburgia ugandensis* extracts against *L. major* and *L. donovani* promastigotes.

Extracts	<i>L. major</i>		<i>L. donovani</i>	
	(IC <sup>50</sup> )	(MIC µg/ml)	(IC <sup>50</sup> )	(MIC µg/ml)
n-hexane	9.95	62.3	8.67	250
Dichloromethane	15.45	62.3	9.18	500
Ethyl acetate	93.65	125	20.79	500
Methanol	Not active	Not active	Not active	Not active
Pentostam	139.31	250	28.41	500
Amphotericin B	2.38	62.3	49.90	250

hexane had the lowest viability followed by ethyl acetate. The viability of promastigotes was below twenty percent at concentrations above 250 µg/ml while at 1000 µg/ml all except methanol had viabilities of 0%.

*Leishmania donovani* promastigotes treated with hexane, dichloromethane and ethyl acetate showed low viability ( $p < 0.05$ ) compared to those treated with methanol. There was no significant difference ( $p > 0.05$ ) in the percentage viabilities of *L. donovani* treated with three extracts compared to pentostam and amphotericin B.

In order to determine the minimum inhibition concentration (MIC), *L. major* promastigotes (106 parasites/ml) were maintained in culture in the presence of test extract that were serially diluted from 1000 to 0 µg/ml. Cell growth was evaluated daily by assessment of turbidity in order to evaluate MIC. The lowest concentration of the test compound that prevented the growth of *L. major* parasites *in vitro* was considered as the MIC. Similarly, activity of *Warburgia ugandensis* extracts against *Leishmania donovani* was investigated. The IC<sup>50</sup> values were computed by entering the optical density readings in a preset MS excel template. The parasites were incubated in varying concentrations of the drugs and the negative control.

#### Cytotoxicity of extracts of *Warburgia ugandensis* to macrophages

The percentage cytotoxicity of the hexane, dichloromethane, ethyl acetate and methanol extracts were determined at a concentration of 1000 µg/ml. The percentage cytotoxicity of the samples were lower than ( $p < 0.05$ ) that of amphotericin and pentostam. Amphotericin B was toxic at 15 µg/ml (8.85%), whereas pentostam was toxic up to 31.25 µg/ml (12.68%). The fractions were not toxic to macrophages at concentrations below 1000 µg/ml.

#### Cytotoxicity of samples of *Warburgia ugandensis* to VERO cells

The IC<sup>50</sup> values of the cytotoxicity of the samples to VERO cells showed that hexane was less toxic ( $p < 0.05$ )

compared to pentostam. However, dichloromethane and ethyl acetate were more toxic (had lower IC<sup>50</sup>;  $p < 0.05$ ) compared to amphotericin and pentostam. The MIC values revealed that concentrations of below 31.25 µg/ml were not toxic for all the samples except methanol which did not produce tangible results. There was no significant difference ( $p > 0.05$ ) between the MIC of hexane, dichloromethane and amphotericin B. However, ethyl acetate had a higher ( $p < 0.05$ ) MIC value compared to amphotericin B.

#### Stimulation of nitric oxide production by *Warburgia ugandensis* extracts in cell free cultures

The nitric oxide production in supernants of macrophage culture treated with hexane, dichloromethane, ethyl acetate and methanol samples were determined using a representative standard curve for samples at concentrations between 0 and 1000 µg/ml. No significant NO levels ( $p > 0.05$ ) were produced compared to the negative control. None of the samples had Optical Density (OD) readings of more than 0.10 indicating that less than 2 µm of NO was produced compared to negative controls that produced similar levels.

#### *In vitro* activity of *Warburgia ugandensis* extracts to *L. major* amastigotes

Different concentrations of samples of hexane, dichloromethane, ethyl acetate and methanol were tested for their efficacy against amastigotes in macrophages. The hexane and dichloroethane samples had the same MIC value with the pentostam (125 µg/ml) while ethyl acetate and amphotericin B had MIC value of 250 µg/ml.

In order to determine the extent of infection, the number of infected macrophages and the number of amastigotes per 100 macrophages was determined. The association indices were determined by multiplying the percentage of infected macrophages by the number of parasites per infected cell. Association indices were the number of parasites that actually infected the macrophages (Table 2). The number of *L. major* and *L. donovani* amastigotes

**Table 2.** Activity of extracts of *W. ugandensis* on amastigotes of *L. major* and *L. donovani*.

Extract	Hexane		Dichloromethane		Ethyl acetate		Methanol		Pentostam		Amphotericin		RPMI	
	Number of amastigotes													
Conc	LM	LD	LM	LD	LM	LD	LM	LD	LM	LD	LM	LD	LM	LD
100µg/ml	2	54	83	68	56	76	80	175	28	4	4	8	546	548
50µg/ml	8	138	185	216	82	181	106	223	5	7	13	12		
25µg/ml	20	202	245	279	137	276	221	351	11	11	210	80		

Adherent macrophages were infected with *L. major* promastigotes at a parasite/macrophage ratio of 6:1, and incubated at 37°C in 5% CO<sub>2</sub> for 4 hours. The medium and drug were added at different concentrations and were replenished daily for 3 days. After 5 days the monolayers were washed with PBS at 37°C, fixed in methanol and stained with 10% Giemsa solution. The number of amastigotes was determined by multiplying the percentage of infected macrophages by the number of parasites per infected cell.

in macrophages treated with hexane, dichloromethane, ethyl acetate, and methanol were significantly lower ( $p < 0.01$ ) compared to the negative control. There was no significant difference ( $p > 0.05$ ) in the number of *L. major* amastigotes in macrophages treated with the hexane extract compared to pentostam and amphotericin B at concentrations of 25 to 100µg/ml.

#### Stimulation of nitric oxide production by *W. ugandensis* extracts in macrophages infected with *Leishmania* parasites

The nitric oxide production in macrophage cultures infected with amastigotes treated with hexane, dichloromethane, ethyl acetate and methanol samples were determined using a representative standard curve for samples at concentrations between 0 and 1000 µg/ml. There was no significant difference in the levels of NO produced by macrophages treated with the extracts ( $p > 0.05$ ) compared to the negative control. None of the samples had OD readings of more than 0.10 indicating that less than 2 µm of NO was produced compared to negative control.

## DISCUSSION

This study examined the antileishmanial activity of extracts of *W. ugandensis*. On the overall, the hexane extract showed very good activity against *L. major* and *L. donovani* parasites. The results scientifically substantiate the claim made by Kenyan traditional practitioners that *W. ugandensis* stem barks have antileishmanial effects. The activity of *W. ugandensis* was at concentrations that were not cytotoxic to macrophages and were less toxic to VERO cells compared to Pentostam and amphotericin B.

The antimicrobial effects of *W. ugandensis* have previously been demonstrated. A recent study in Kenya showed its activity against soil pathogens namely *Fusarium oxysporum*, *Alternaria passiflorae*, and *Aspergillus niger* (Rugutt et al., 2006). Olila et al. (2001) have demonstrated that this plant has both antibacterial and antifungal activities. However, the antiparasitic effects of this

plant are limited since previous studies have indicated no activity against trypanosomes (Kioy et al., 1998) and *Giardia lamblia* (Johns et al., 1995).

The findings in this study indicate that hexane extracts of *W. ugandensis* have good antileishmanial activity. The IC<sub>50</sub> values of the hexane extract against *L. major* promastigotes (9.95) was better than that of Pentostam (139.3) a well known first line drug and comparable to amphotericin B (2.38). It is noteworthy that Pentostam is usually not very effective in vitro since it is processed in vivo before it acts against *Leishmania* parasites. On the other hand, the effectiveness of the hexane extract is supported by the fact that the minimum inhibition concentration of 62.3 µg/ml is the same as that of amphotericin B and lower than that of Pentostam (250 µg/ml). Similar results were obtained for activity against *L. donovani* promastigotes. Similarly the extracts significantly ( $p < 0.01$ ) reduced the infectivity of *L. major* amastigotes in macrophages. There was a hundred fold decrease in the number of *L. major* amastigotes in macrophages treated with the hexane extract compared to the negative control. The antileishmanial activity of dichloromethane and ethyl acetate extracts was lower than that of the hexane extract although better than that of the methanol extract which showed limited or no activity against both promastigotes and amastigotes. It is possible that the active components in *W. ugandensis* have low polarity since the highly polar solvent (methanol) did not seem to extract compounds with activity against *Leishmania* parasites. It is interesting to note that the *W. ugandensis* fractions did not lead to an increase in the levels of production of nitric oxide by macrophages. This point to the fact that the extracts may be acting directly on the parasites rather than stimulating the immune system to counteract the parasites.

Further studies should investigate the active compounds in the hexane, ethyl acetate and dichloromethane extracts since they could be useful templates for drugs against leishmaniasis. *In vivo* studies should be carried out to test the efficacy of the extracts against the leishmaniasis of other species in susceptible animal models at various stages of disease progression.

## Conclusion

This study scientifically demonstrates the antileishmanial potential of *W. ugandensis*.

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