

Inhibition of *Trypanosoma brucei brucei* by Extracts from *Waltheria indica* L. (Sleepy Morning)

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ABSTRACT

The study was conducted to evaluate the inhibitory activity of polar and non-polar extracts from *Waltheria indica* against *Trypanosoma brucei brucei*, the causative parasite of trypanosomiasis, for the first time. The parasites *Trypanosoma brucei brucei* were cultured using 96 well micro titer plate and maintained at 37°C. About 20-25 parasites per microscope field were dozed with 1, 2 and 4 mg mL⁻¹ of the extracts of the plant. Two controls were set up; one is Berenil (the reference drug), which was used as positive control and a negative control group without extract/Berenil. Results showed that the ethanol extract was the most active with Inhibitory Concentration (IC₅₀) value of 0.5-1 mg mL⁻¹ while the petroleum ether extract was moderate against the trypanosome parasites with IC₅₀ value of 3.8-4.0 mg mL⁻¹. The results also showed that the parasites survived more than 4 h when no plant extract/berenil was present. Phytochemical screening revealed a range of compounds including, alkaloids, terpenes, flavonoids, tannins, glycosides, steroids, among others, which could be responsible for the trypanocidal activity of the extracts. Further studies including *in vivo* assays for the determination of efficacy and toxicity are planned and will be carried out.

Key words: Trypanocidal activity, *Waltheria indica* extracts, *in vitro* study

INTRODUCTION

African trypanosomiasis remains a disease with unsatisfactory medical control (Maikai *et al.*, 2008). The control of trypanosomiasis continues to rely principally on chemotherapy and chemoprophylaxis using the salts of the three compounds, diminazene; an aromatic diamidine; homidium, a phenanthridine and isometadim, phenanthridine-aromatic amidine (Anene *et al.*, 2001). However, the therapeutic and prophylactic use of trypanocides is beset by numerous limitations, including toxicity and the development of resistance by the parasites (Gutteridge, 1985). The emergence of drug resistance trypanosome strains is considered a very serious problem in trypanosomiasis control, particularly for the resource-poor at risk populations and farmers in Africa. Recent surveys in Eastern and Southern Africa (Ndungu *et al.*, 1999) and in West Africa (McDermott *et al.*, 2000; Maikai *et al.*, 2007) have shown that the prevalence of trypanocidal drug resistance might even be higher than hitherto expected. The limited availability and affordability of pharmaceutical medicines emphasizes the need for research into a more comprehensive, formidable and cheaper sources of trypanocide. It is estimated that some 20,000 species of higher plants are used medicinally throughout the world (Tagboto and Townson, 2001). Plants have

provided the basis for traditional treatment for different types of diseases and still offer an enormous potential source of new chemotherapeutic agents. Plants present a spectrum of biological compounds with activities against virus, cancer and parasites. Plants contain compounds mainly secondary metabolites such as alkaloids, Glycosides, flavonoids, terpenes and coumarins (Rates, 2001) which have been reported to provide better and cheaper alternative drugs against parasites (Nwude and Ibrahim, 1980; Freiburghaus *et al.*, 1996; ITDG and IIRR, 1996; Adewummi *et al.*, 2001; Nok, 2005).

Waltheria indica L. (sleepy morning) is a short-lived shrub that is popular among traditional healers, here in Sokoto State, in the treatment of skin diseases, malaria and typhoid fever, sickle cell anaemia and epilepsy. In addition the aqueous extract of the plant was shown elsewhere (Bala *et al.*, 2009) to possess an *in vitro* trypanocidal activity. However, no scientific data has been published to show the trypanocidal effects of various extracts of the plant.

Therefore this study is designed to determine the inhibitory effects and phytochemical analysis of polar and non polar extracts of *Waltheria indica* on *Trypanosoma brucei brucei* parasites *in vitro*. This is the first report on the trypanocidal activity of the various extracts of the plant.

MATERIALS AND METHODS

Collection of the plant: The plant used in this study was collected in October 2007 from Dabagi farm of the Usmanu Danfodiyo University, Sokoto and transported to the Botany Unit of the Usmanu Danfodiyo University, Sokoto, for identification with the help of a taxonomist, A.M. Umar. A voucher specimen of the plant was deposited in the herbarium of the Department where it was identified.

Preparation of the plant extracts: Whole plant of *Waltheria indica* was cut into pieces, air-dried (under shade to avoid destruction of the active components) at room temperature and pulverized using mortar and pestle. Various extracts of the plant part were prepared in a separating funnel using activity guided fractionation method as described by Hostettamn *et al.* (1995). In this method, 40 g of the pulverized plant part was dissolved in 50 mL of water and ethanol. It was then left to stand for 24 h after which it was filtered. The residue was discarded and to the water-ethanol filtrate, 50 mL petroleum ether was added and shaken for 30 min in a separating funnel. The petroleum ether extract was separated and evaporated in vacuo at 45°C and the residue was weighed and stored. To the water-ethanol filtrate, 50 mL ethyl acetate was added using the same procedure above and then followed by n-Butanol. Finally, the water-ethanol filtrate was separated, dried, weighed and stored until required.

Test organisms: *Trypanosoma brucei brucei* were obtained from stabilates maintained at the Nigerian Institute of Trypanosomiasis and Onchocerciasis Research (NITOR) Vom, Plateau State. The parasites were maintained in the laboratory by continuous passage in rats until required. Passage was considered necessary when parasitaemia reaches a range of 16-32 parasites per field (usually 3-5 days post infection). In passaging, 1×10^8 parasites in 0.1-0.2 mL blood/PBS solution was introduced intraperitoneally into clean rats acclimatized under laboratory condition for 1 week.

***In vitro* study of extracts of *Waltheria indica*:** Assessment of *in vitro* trypanocidal activity was performed in triplicates in 96 well micro titer plates (Atawodi *et al.*, 2003), but with slight modification. The 0.5 g of each extract was dissolved in 25 mL distilled water to obtain 20 mg mL⁻¹

concentration. Using the same process two other concentrations of 10 and 5 mg mL⁻¹ were also formed, giving three different concentrations for each extract. The 20, 10 and 5 mg mL⁻¹ concentrations of each plant extract was prepared in triplicates.

Twenty microliter of blood containing about 20-25 parasites per field obtained as described above, was mixed with 5 µL of each extract concentration to produce effective test concentrations of 4, 2 and 1 mg mL⁻¹, respectively. To ensure that the effect monitored was that of the extract alone, two sets of control were set up. First Diminazene aceturate (Berenil-a commercial trypanocide) was used as positive control and then blood suspended in normal saline was used as a second (negative) control. Berenil was prepared in the same concentration as the extracts (445 mg Diminazene aceturate + 555 mg Antipyrine, Eagle Chemical Company LTD, Ikeja, Nigeria).

Each of the test mixtures was incubated for 5 min in closed Eppendorf tubes maintained at 37°C. Two microliter of test mixture was placed on separate microscope slides and covered with a cover slip and the parasites observed for a total duration of 60 min.

The concentration at which 50% of the trypanosomes were cleared by each treatment was recorded and taken as the IC₅₀ value of the respective extract.

Phytochemical analysis: Phytochemical screening of the ethanol and petroleum ether extract of *Waltheria indica* was carried out to determine the presence of secondary metabolites using standard procedures (El-Olemy *et al.*, 1994; Harborne, 1973). Flavonoids were determined by acidifying 2 mL of each extract by adding equal volume of 0.1 M HCl. 0.5 mL of 0.5 M NaOH was added to the acidified extract. A yellow colour indicates the presence of flavonoids (El-Olemy *et al.*, 1994; Harborne, 1973). The presence of saponins was assessed by vigorously mixing 1 mL of each extract in a corked test tube. Persistent froth, lasting for several minutes was indicative of the presence of saponin. The presence of saponin was confirmed by shaking 2 mL of diluted fraction with 1 mL of olive oil for about half a minute. A thick white emulsion confirmed the presence of saponin (El-Olemy *et al.*, 1994). Tannins were determined by using 5% ferric chloride solution which was added drop wise to 3 mL of diluted extract. The presence of condensed tannins was indicated by green-black colouration, while blue-black was indicative of hydrolysable tannins (El-Olemy *et al.*, 1994). Triterpenes and Steroids were assessed by the Liebermann and Burchard Method as done by El-Olemy *et al.* (1994). To about 1 mL of the fraction was added equal volume of concentrated H₂SO₄ and acetic anhydride. Green-pink to purple violet indicated the presence of triterpenes. The presence of sterols is indicated by pink to brown/yellowish brown colour. Blue-green colour is indicative of both sterols and triterpenes. The presence of Alkaloids was determined by stirring 1 mL of each extract with 5 mL of 10% aqueous hydrochloric acid on a steam bath for 20 min, which was allowed to cool and was later filtered. One microliter of the filtrate was treated with drops of Mayer's reagent. Appearance of creamy precipitate indicated the presence of alkaloids. Glycosides were determined by adding 10 cm³ of 50% H₂SO₄ to 1 cm³ of each extract in a test tube. The mixture was heated in boiling water for 15 min. The 10 cm³ of Fehling's solution was added and the mixture was heated again in a boiling water bath. A brick-red precipitate indicated the presence of glycosides (El-Olemy *et al.*, 1994).

Statistical analysis: Percentage inhibition of parasite population caused by each treatment was calculated with respect to the parasite-laden control without treatment as number of parasites in the test well divided by the number of parasites in the control multiplied by a hundred. Final

percentage inhibition for each concentration was obtained as mean of percentage inhibition for that concentration. Data in Table 1 were evaluated statistically with one-way ANOVA followed by Duncan's Multiple Range test.

RESULTS

Inhibitory activity of polar and nonpolar extracts of *Waltheria indica* analyzed against *Trypanosoma brucei brucei* parasites *in vitro* are presented in Table 1.

The results showed that at 1.0 mg mL⁻¹ only the ethanol extract of the plant had effect on the trypanosome parasites, producing 93.33% inhibition of trypanosome parasites and comparing favourably with the standard drug of choice Berenil which produces an inhibition of 98.4%.

At 2.0 mg mL⁻¹, ethanol extract inhibited the trypanosomes parasites with a percentage of 96.2. The other extracts had no such effect on the parasites while Berenil 100% inhibited the trypanosome parasites at this concentration.

At 4.0 mg mL⁻¹, 52.1 and 98.8% inhibition was found for petroleum ether and ethanol extracts respectively. Berenil was able to clear the parasites at this concentration with 100% inhibition.

The IC₅₀ value for the ethanol extract was found to be between 0.5-1 mg mL⁻¹ while that of the petroleum ether extract was between 3.8-4 mg mL⁻¹.

Results also showed that after 5 min incubation in Eppendorf tubes maintained at 37°C, the trypanosome parasites survived for about 4 h when no extract/Berenil was present.

The results showed the ethanol extract to be the most effective against *Trypanosoma brucei brucei* parasite on all concentrations tested while the petroleum ether extract was moderate, active only at the highest concentration of 4 mg mL⁻¹.

The result of the qualitative phytochemical screening of the ethanol extract of *Waltheria indica* showed an appreciable presence of tannins, terpenes, flavinoids, phenols and glycosides. Alkaloids and steroids were mildly present. In the petroleum ether extract glycosides and steroids were highly present while tannins, terpenes and flavonoids were mildly detected. Saponins and anthraquinones were not detected in both extracts (Table 2).

Table 1: Inhibitory activity of various extracts of *Waltheria indica* against *Tyranosoma brucei brucei* parasites *in vitro*

Treatments	Inhibition (%) of parasite population caused by treatment at concentration of		
	1.0	2.0	4.0
	(mg mL ⁻¹)		
n-Butanol	0.00±0.00	0.00±0.00	0.00±0.00
Ethanol	93.33±3.52*	96.2±2.16**	98.8±1.16**
Ethyl acetate	0.00±0.00	0.00±0.00	0.00±0.00
Pet. Ether	0.00±0.00	0.00±0.00	52.1±2.45*
Berenil®	98.4±1.20*	100.00±0.00**	100.00±0.00**

Each value represents mean±SEM of 3 replicates. *p<0.05; **p<0.01

Table 2: Result of qualitative phytochemical analysis of the ethanol and petroleum ether extracts of *Waltheria indica*

Extract	Alkaloids	Tannins	Saponins	Terpenes	Flavonoids	Glycosides	Anthraquinones	Phenols	Steroids
Ethanol	+	++	-	++	++	++	-	++	+
Petroleum ether	-	+	-	+	+	++	-	-	++

-: Absent; +: Mildly present; ++: Highly present

DISCUSSION

Natural products isolated from higher plants have provided novel clinically active drugs which are one of the keys to the discovering of naturally occurring therapeutic agents through bioassay-guided fractionation and purification procedures. When fractionation or purification enhances activity, it often leads to the discovery of new active molecules. On the other hand if it leads to reduction in activity, the constituents are synergistic, as such drugs are to be formulated at the stage of highest activity. In this study, *W. indica* was subjected to fractionation and the bioactive constituents were found to be in the ethanol extract, the polar portion. This had antitrypanosomal activity at all concentrations tested. The trypanocidal activity of the polar portion of *Waltheria indica* seen in this study is in agreement with reports from several researchers (Kamanzi *et al.*, 2004; Ogbunugafor *et al.*, 2007; Abdel-Sattar *et al.*, 2009). However, the results were in disagreement with the findings of Hoet *et al.* (2004), Agbedahunsi *et al.* (2006) and Ene *et al.* (2009) who respectively found the ethyl acetate, chloroform and methylene chloride extracts of some plant materials to have the most antitrypanosomal activity.

The observation that different solvent extracts of plants have different trypanocidal activities is in line with the report of Freiburghaus *et al.* (1996), who clearly indicated that different solvent extracts of the same plant may exhibit different trypanocidal activity, just as extracts of different parts of the same plants. Therefore, different solvent extracts of plants should be tested separately for medicinal purposes. The petroleum ether extract of *Waltheria indica* was found to be moderately active against the trypanosome parasites, an indication that it may also contain some active constituents that have trypanocidal potential. Although the concentration of the extracts used in this study was high (up to 20 mg mL⁻¹), the morphology of the red blood cells was maintained while that of the trypanosome parasites was affected, when compared to the parasite laden control. This is an indication that a higher trypanocidal activity may be obtained by increasing the concentration of the extracts.

The trypanocidal potential of the extracts was further confirmed by phytochemical analysis which revealed a range of compounds that could be responsible for the activity. The phytochemical analysis revealed both extracts to contain tannins, terpenes, flavonoids, steroids, phenols and glycosides. Terpenes, steroids and alkaloids have been reported to have high antitrypanosomal effects (Hoet *et al.*, 2007; Ene *et al.*, 2009). Anthrquinones which was absent in both extracts was reported to have activity against *T. congolense* (Nok *et al.*, 1996). Some literatures have reported that flavonoids had antitrypanosomal activity (Tarus *et al.*, 2002). Therefore this study has shed new light, as an added data on our earlier findings (Bala *et al.*, 2009), on the trypanocidal activity of the ethanol and petroleum ether extracts of *waltheria indica*.

The mechanism by which the extracts exhibit trypanocidal action is not immediately known. 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. It is also known that some agents act by binding with the kinetoplast DNA of the parasite. Many others too act by static action affecting growth and multiplication of trypanosomes.

In conclusion, results have clearly shown that *Waltheria indica* possesses anti-trypanosomal activity *in vitro*. The trypanocidal property was found to be higher in the polar portion and moderate, at the highest test concentration, in the non polar part. The antitrypanosomal activity

of the extracts is not surprising because earlier, Bala *et al.* (2009) has shown the aqueous extract to possess *in vitro* trypanocidal activity. Phytochemical screening reveals a range of compounds, alkaloids, terpenes, flavonoids, tannins, glycosides, steroids, among others, which could be responsible for the trypanocidal activity of the extracts. Further studies are in progress to identify the active constituents, toxicity and the trypanocidal activity of the extracts *in vivo* using animal models.

ACKNOWLEDGMENT

The authors wish to thank Head and Technicians of Department of Biochemistry, Usmanu Danfodiyo University, Sokoto for creating such an enabling environment in their lab for this work to be conducted. We also wish to thank Professor N.S. Naqvi for dedicating his pension as postgraduate scholarship which provided financial support for this study.

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