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IN VITRO AND *IN VIVO* ANTI-TRYPANOSOMAL ACTIVITIES OF METHANOL EXTRACT OF *AZADIRACHTA INDICA* STEM-BARK

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Abstract

Background: Current chemotherapeutic agents for the treatment of African trypanosomiasis have become largely ineffective, necessitating the search for alternative compounds. The objective of this study was to evaluate *in vitro* anti-trypanosomal activities of methanol extracts of parts of *Azadirachta indica* against *Trypanosoma brucei rhodesiense, Trypanosoma brucei brucei* and *Trypanosoma evansi* and establish the *in vivo* efficacy of the most active extract.
Materials and methods: Maceration of powdered leaves, stem bark and root bark of the plant in methanol afforded three extracts. *In vitro* assays were carried out with the extracts on the three trypanosome strains in 96-well microtitre plates at concentration ranges of 4000 - 1000µg/ml. The most active extract was assayed *in vivo* using *Trypanosoma brucei rhodesiense* infected Swiss albino mice at doses of 100, 200 and 400 mg/kg body weight. Melarsoprol and suramin served as positive controls. The infected untreated group served as the negative control. Parasitaemia levels, packed cell volume, body weight changes and mean survival period of all groups were monitored throughout the experimental period.
Results: Methanol extract of the stem bark of *A.indica* was most active *in vitro* against all the three trypanosome strains (MIC values of 9.93±1.88, 16.25±0.92 and 9.97±0.44µg/ml for *T. b. rhodesiense, T. b. brucei* and *T. evansi*, respectively). The extract showed optimum activity at 400 mg/kg and was comparable to the positive control groups. Parasitaemia levels were kept at a significantly low level ($p < 0.05$) by the extract compared to the negative control. Notably, there was no significant difference ($p>0.05$) in mean survival time of mice treated with the extract at 400 mg/kg and the positive controls.
Conclusion: *In vitro* and *in vivo* anti-trypanosomal activities of the methanol extract of *A. indica* stem bark could be attributed to the presence of constituents of moderate polarity.

Key words: Anti-trypanosomal activity, *Azadirachta indica, Trypanosoma brucei brucei, Trypanosoma brucei rhodesiense, Trypanosoma evansi*

Introduction

African trypanosomiasis is caused by single-celled protozoa of the genus *Trypanosoma*. *Trypanosoma brucei, T. b. gambiense* and *T. b. rhodesiense* are the main causative agents of human African trypanosomiasis whereas animal African trypanosomiasis is caused by *T. b. brucei, T. congolense, T. vivax, T. evansi* and *T. equiperdum* (Brun, Hecker, & Lun, 1998; Patrick et al., 2012; Swallow, 2000). African trypanosomiases impact negatively on the entire economy of Africa by weakening the health of both humans and animals (John, Rachid, Damian, Glyn, & Stephen, 2012). Parasite resistance to the existing chemotherapeutic agents compounded by the poor prospect for a vaccine due to antigenic variation of the parasite has spurred the search for alternative compounds for African trypanosomiasis (Florencia & Stefan, 2011).

However, there has been no significant resistance observed in clinical isolates and laboratory selection for some agents such as suramin. A possible explanation could lie in the fact that suramin unselectively inhibits enzymes and receptors, and therefore could inhibit multiple targets (Fairlamb, 2003). The current study aimed at investigating *in vitro* and *in vivo* anti-trypanosomal activities of methanol extracts of *A. indica* plant parts.

Azadirachta indica has been used as traditional medicine for the management of various diseases for decades due to its medicinal properties (Brototi & Kaplay, 2011; Kumar, Debasis, Goutam, & Chandra, 2010). Extracts of *A. indica* have been reported to exhibit various pharmacological activities (Kumar et al., 2010). In the current study, treatment of *T. b. rhodesiense* infected Swiss white mice with the methanol extract of *A. indica* stem bark revealed that the extract was efficacious at a dose level of 400 mg/kg.

Materials and Methods

Plant collection and extraction

Leaves, stem- and root-barks of *A. indica* were collected from Pwani University (Kilifi, North coast of Kenya). The plant species was identified by a taxonomist and a voucher specimen (Specimen No. EN/05/2012) deposited at the herbarium of the department of Pharmacy & Complementary/ Alternative Medicine, Kenyatta University and also at the East African Herbarium, National Museums of Kenya, Nairobi. The air dried plant parts were ground into fine powders followed by extraction at room temperature by maceration for 24 hours in methanol. The extracts were partitioned between water and dichloromethane (DCM). The DCM phases were combined and concentrated *in vacuo*.

Trypanosomes

Cryopreserved stabilates of *Trypanosoma b. rhodesiense* (KETRI 3438), *T. b. brucei* (EATRO 2400) and *T. evansi* (KETRI 2454) were obtained from the Biotechnology Research Institute, Kenya Agricultural and Livestock Research Organization, (KALRO) trypanosome bank. The stabilates were thawed at room temperature, suspended in cold phosphate-saline-glucose (PSG) buffer pH 8.0 and injected intraperitonially into immunosuppressed donor mice for multiplication. At the first peak of parasitaemia, donor mice were anaesthetized using carbon dioxide and blood collected from the heart of the mice using a syringe containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant. The parasitaemia count was quantified using a haemocytometer and the blood was diluted appropriately with PSG buffer to obtain the required inoculum dose of 1×10^4 trypanosomes/ml.

In vitro assays for anti-trypanosomal activity

The *in vitro* antitrypanosomal assays were carried out in 96-well microtitre plates according to the method by Räz *et al.*, (1997). The first stock solutions of the extracts were prepared in distilled water for the water-soluble samples at 20 mg/ml or in dimethyl sulfoxide (DMSO) (at 1 mg/100 µl) for the water-insoluble samples. The extracts were assayed on the three trypanosome strains at concentration ranges of 4000 - 1000µg/ml. Each plate was examined with an inverted microscope to determine the minimum inhibitory concentration (MIC) which is the concentration at which no cell with a normal morphology and/or motility was found in comparison to the negative control cultures (Githua et al., 2010).

Experimental animals

Randomly selected healthy female Swiss white mice weighing between 20 - 25 g were obtained from Biotechnology Research Institute, KALRO small animal breeding unit. They were dewormed using injectable ivermectin at 0.1ml per mouse and were acclimatized for 14 days during which they were maintained on a diet of commercial mice pellets (mice pellets®, Unga Ltd, Nairobi, Kenya) and water was provided *ad libitum*. All experimental protocols and procedures used on animals during the study were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Biotechnology Research Institute, KALRO.

Determination of acute toxicity

Twenty mice were randomly placed into four groups of five animals each. Each group received treatment of the following doses of the methanol extract of the stem bark intraperitoneally, daily for four days: Group i (100 mg/kg body weight (bwt); Group ii (200 mg/kg bwt); and Group iii (400 mg/kg bwt). Group iv was given water only. Animals were initially observed closely for a period of 10-15 minutes following administration of the test drug for signs of acute toxicity. The mice were then closely monitored throughout the 4 days of administration and two weeks of experiment. Changes in posture, breathing, food intake and water consumption patterns of the treated mice were noted on a daily basis. Excessive

weight loss and changes of packed cell volume (PCV) of more than two-fold compared with the control group of mice over the 2 weeks period was considered a key indicator of declining health due to drug toxicity (Mbaya, Ibrahim, God, & Ladi, 2010).

Determination of *in vivo* efficacy

Thirty five mice were randomly divided into seven groups of five mice each. Each experimental mouse was intraperitonially injected 0.2 ml of PSG buffer containing an inoculum dose of 1×10^4 trypanosomes/ml of *T. b. rhodesiense* (KETRI 3438) from donor mice. The mice were then treated as follows: Group i; i.p. treatment of 100 mg/kg bwt of extract, 24 hours post-infection (PI) for four days; Group ii ; i.p. treatment with 200 mg/kg bwt of extract from 24 hours PI for four days; Group iii; i.p. treatment with 400 mg/kg bwt of extract from 24 hours PI for four days; Group iv; intraperitoneally treated using suramin (5 mg/kg bwt) from 24 hours PI for three days (positive control I), Group v; intraperitoneally treated using melarsoprol (3.6 mg/ml) from 24 hours PI for three days (positive control II), Group vi; infected and given water *ad-libitum* with no extract (negative control), Group vii; uninfected and untreated (blank group). Group vii was included in the study for purposes of reference especially on the survival of the mice throughout the experimental period (60 days). Determination of *in vivo* efficacy was based on the changes in the levels of parasitaemia, packed cell volume (PCV) and weights of the animals during the experimental period.

Parasitaemia examination

The level of parasitaemia was determined daily for five days after the first treatment, then every two days for the remaining days of the experimental period. A drop of blood was collected from the mouse tail, placed on a clean slide, covered with a cover slip and viewed under the microscope at x400 magnification using the method of Herbert and Lumsden (1976).

Determination of packed cell volume (PCV)

Packed cell volume (PCV) was determined by bleeding tail vein of mice and filling three-quarters full heparinized capillary tubes with 50-60 µl of the blood. The sealed tubes were centrifuged at 10000 rpm for 5 minutes in a haematocrit centrifuge, and the height of the red blood cell column was measured by use of haematocrit reader. The percentage of the total blood volume occupied by red blood cell mass was considered as the PCV.

Mean Survival Time

The survival time for each mouse was recorded by daily checking the cages for mortality. The number of dead mice was recorded throughout the experimental period. The average survival time was then calculated.

Data Analysis

Statistical Package for Social Science (SPSS) version 21 was used for data analysis and the values obtained were expressed as mean ± standard error of mean (SE). The significant differences between variables were compared by one-way ANOVA followed by Student Newmann Keul's test (SNK). P values less than 0.05 were considered significant.

Results

The *in vitro* activities were classified according to Hoet *et al*.(Hoet et al., 2004). It emerged that the stem bark extract was the most active exhibiting anti-trypanosomal effects against all the three strains used in the study (Table 1).

Table 1: *In vitro* anti-trypanosomal activity (MIC) of methanol extracts of *A. indica*

Plant Part/	MIC ± SE (µg/ml) against		
Compound	*T. b. rhodesiense*	*T. b. brucei*	*T. evansi*
Leaf	51.98±1.21	48.17±0.11	22.13±0.14
Stem bark	9.93±1.88	16.25±0.92	9.97±0.44
Root bark	23.50±0.07	18.38±0.25	32.70±0.09
Melarsoprol	0.003 ± 0.001	0.005 ± 0.002	0.003 ± 0.001
Suramin	1.54 ± 0.09	1.66 ± 0.10	1.51 ± 0.13

Acute toxicity studies

No animal mortality was observed during the 14 days of experimentation. There were no visible signs of acute toxicity among the animals treated with the extract at the doses used in the study.

Effect of the methanol extract of *A. indica* on parasitemia of *T. b. rhodesiense* infected mice

There was a delay in the commencement of parasite appearance in the extract treated groups (Table 2). The optimum activity of the extract was obtained at 400 mg/kg, at which there was no significant difference ($p>0.05$) with positive control groups. Compared with the negative control, the 400 mg/kg extract kept parasitaemia at a significantly low level ($p < 0.05$) throughout the experimental period.

Table 2: Effect of methanol extract of *A. indica* stem-bark on parasitemia of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Parasitemia level (log number)/mL							
	D7	D14	D21	D28	D35	D42	D49	D56
100 mg/kg	3.90 ± 1.76^{ab}	6.48 ± 1.64^{b}	6.00 ± 1.62^{bc}	6.72 ± 1.71^{c}	6.96 ± 1.75^{c}	5.90 ± 1.75^{b}	5.40 ± 1.29^{c}	4.80 ± 1.96^{c}
200 mg/kg	2.88 ± 1.62^{ab}	4.44 ± 1.82^{ab}	5.04 ± 2.07^{b}	5.10 ± 1.09^{bc}	5.08 ± 2.16^{bc}	3.00 ± 1.07^{b}	2.80 ± 0.80^{b}	3.00 ± 1.07^{bc}
400 mg/kg	0.00 ± 0.00^{a}	1.62 ± 0.32^{a}	1.62 ± 0.32^{ab}	1.38 ± 0.14^{ab}	1.68 ± 0.80^{ab}	1.74 ± 0.41^{ab}	1.80 ± 0.18^{ab}	1.74 ± 0.41^{ab}
3.6 mg/kg Mel	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5 mg/kg Sur	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Negative control	6.60 ± 1.66^{b}	8.34 ± 0.11^{b}	8.64 ± 0.29^{c}	8.48 ± 0.28^{c}	8.55 ± 0.28^{c}	9.00 ± 0.00^{c}	–	–

Values are mean ± SEM; SE = standard error; n = 5; D = day; Mel = melarsoprol; Sur = suramin; negative control = infected untreateated; – implies that mice were dead; values with the same superscript(s) down the column do not differ significantly at $p< 0.05$

Effect of extract on packed cell volume of *T. b. rhodesiense* infected mice

There was no significant difference in maintaining the PCV values in the animals treated with the extract at 400 mg/kg and the positive control groups (melarsoprol and suramin) throughout the study period ($p >0.05$). Packed cell volume (PCV) values in the negative control group started declining and remained lower than the values of the extract treated groups (Table 3).

Table 3: Effect of methanol extract of *A. indica* stem-bark on packed cell volume of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Packed cell volume (PCV(%)) of mice							
	D7	D14	D21	D28	D35	D42	D49	D56
100mg/kg	47.00 ± 0.71^{a}	45.40 ± 2.73^{ab}	44.20 ± 4.47^{ab}	43.80 ± 2.99^{ab}	43.00 ± 3.30^{ab}	48.33 ± 3.61^{b}	45.33 ± 4.11^{a}	48.00 ± 0.00^{a}
200 mg/kg	49.80 ± 1.87^{a}	45.60 ± 2.20^{ab}	48.60 ± 2.23^{ab}	50.20 ± 1.11^{b}	46.20 ± 2.87^{ab}	51.00 ± 3.22^{b}	50.67 ± 2.21^{bc}	49.67 ± 3.39^{a}
400 mg/kg	50.00 ± 1.24^{ab}	49.60 ± 1.81^{ab}	48.80 ± 1.24^{ab}	50.40 ± 0.81^{b}	50.00 ± 2.35^{b}	50.40 ± 2.75^{b}	51.20 ± 1.84^{bc}	51.00 ± 1.10^{ab}
3.6 mg/kg Mel	52.40 ± 1.69^{b}	51.80 ± 1.21^{b}	53.20 ± 0.37^{b}	53.60 ± 0.80^{b}	52.80 ± 0.86^{b}	54.80 ± 0.49^{b}	52.80 ± 0.37^{c}	52.90 ± 0.58^{b}
5 mg/kg Sur	52.00 ± 1.05^{b}	52.60 ± 0.51^{b}	51.40 ± 0.51^{b}	52.20 ± 1.16^{b}	53.20 ± 0.86^{b}	55.20 ± 0.73^{b}	54.60 ± 0.75^{c}	53.80 ± 0.80^{b}
Negative control	47.40 ± 1.03^{a}	43.00 ± 1.34^{a}	42.00 ± 1.76^{a}	40.75 ± 4.96^{a}	39.67 ± 2.85^{a}	37.50 ± 2.21^{a}	–	–

Values are mean ± SE; SE = standard error; n = 5; D = day; Mel = melarsoprol; Sur = suramin; negative control = infected untreateated; – implies that mice were dead; values with the same superscript(s) down the column do not differ significantly at $p< 0.05$

Effect of stem-bark extract of *A. indica* on body weight of *T. b. rhodesiense* infected mice

At the optimum dose of 400 mg/kg the extract treated animals and positive control groups showed comparable increment in body weight (Figure 1).

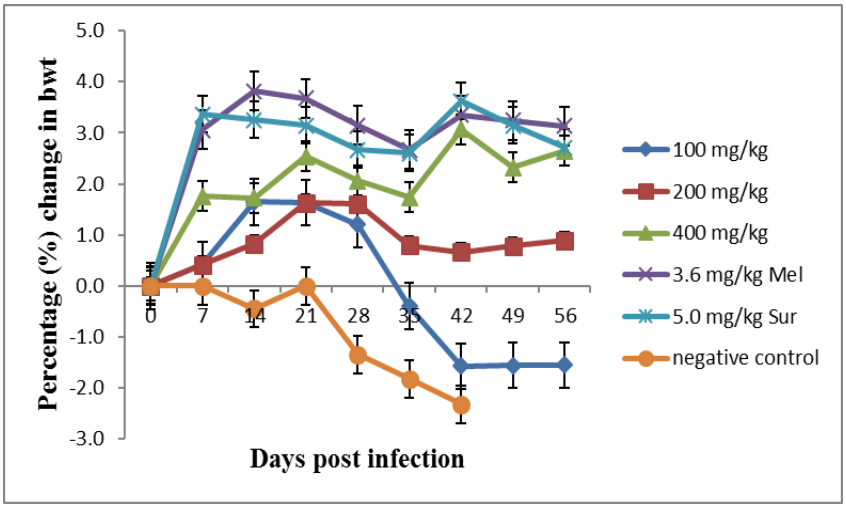

Figure 1: Trends of percentage (%) change in body weight of *T. b. rhodesiense* (KETRI 3438) infected mice treated intraperitoneally with methanol extract of *A. indica* stem bark; values are mean ± SE; n = 5; negative control = infected untreated; Mel = melarsoprol; Sur = suramin

Effect on mean survival time of *T. b. rhodesiense* infected mice

Animals treated with the extract at various dose levels survived for longer time compared to the infected-untreated control (33.00±3.08 days). The survival times between the extract treated animals at higher doses (200 and 400 mg/kg) differed significantly with the negative control group. The mean survival time of animals treated with the extract at 400 mg/kg was comparable to those in positive control groups.

Table 4: Effect of methanol extract of *A. indica* stem-bark on mean survival time of *T. b. rhodesiense* (KETRI 3438) infected mice

Treatment group	Number of mice			Percentage of mice cured	Mean survival time (days ± SE)
	treated	survived	cured		
100 mg/kg	5	1	0	0	46.2 ± 5.44^{ab}
200 mg/kg	5	3	1	20	51.8 ± 5.14^{bc}
400 mg/kg	5	5	4	80	59.6 ± 0.40^{c}
3.6 mg/kg Mel	5	5	5	100	60.0 ± 0.00^{c}
5 mg/kg Sur	5	5	5	100	60.0 ± 0.00^{c}
Negative control	5	0	0	0	33.0 ± 3.08^{a}
Blank	5	5	5	100	60.0 ± 0.00^{c}

SE = standard error; Mel = melarsoprol; Sur = suramin; negative control = infected untreated; blank = uninfected untreated; values with the same superscript(s) do not differ significantly at $p<0.05$.

Discussion

Anti-trypanosomal activity of *A. indica* reported in the present study is comparable to a report by Mbaya *et al.* (Mbaya et al., 2010) which showed that *A. indica* stem bark possesses *in vitro* trypanocidal effect against *T. b. brucei*. A study on the leaf extract of *A. indica* by Githua *et al*. (2010) showed that the extract was active *in vitro* against procyclic forms of *T. b. rhodesiense*.

Results from *in vivo* efficacy study indicated that at a higher dose of 400 mg/kg, the methanol extract of *A. indica* stem bark maintained parasitaemia in *T. b. rhodesiense* infected mice at significantly lower levels as compared to the negative control indicating the presence of anti-trypanosomal constituents in the extract. However, a steady rise in parasitaemia levels was observed in animals treated with the extract at lower doses of 100 and 200 mg/kg from D14 to D56, indicating that the ability of the extract to control parasitaemia levels of infected mice was dose-dependent. A similar trend was observed in the ability of the extract to extend survival period of infected mice. These observations demonstrate that the anti-trypanosomal activity of the extract could be improved by using a higher dose or isolated active compounds in the extract.

The extract significantly reduced weight loss and decline in packed cell volume (PCV) associated with parasitaemia. Packed cell volume and weight loss are common and critical features in the pathogenesis of African

trypanosomiasis contributing to morbidity and mortality (Kagira, Thuita, & Ngotho, 2006). Infected mice treated with *A. indica* extract in the current study showed significantly higher levels in PCV compared to the negative control (infected untreated) group which can be ascribed to an enhanced resistance of erythrocyte haemolysis (Kagira et al., 2006).

These findings are in agreement with a previous study which reported that *A. indica* exhibited encouraging *in vivo* trypanocidal activity with a reduction in the level of *T. brucei* parasitemia in mice (Mbaya et al., 2010). A similar study (Ngure et al., 2009) revealed that the aqueous extract of *Azadirachta indica* was active against *Trypanosoma brucei rhodesiense*, indicating that polar constituents of the stem bark could be contributing to the antitrypanosomal effects of the plant part.

Conclusion

The current study established that the methanol extract of *A. indica* stem bark possess both *in vitro* and *in vivo* antitrypanosomal activity against *T. brucei rhodesiense*. Isolation and characterization of constituents of the extract, which is currently on-going research, could yield a potential antitrypanosomal lead compound.

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Conflict of Interest: Authors declare that there is no conflict of interest

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