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Full Length Research Paper

Screening of *Vernonia amygdalina* and *Hymenocardia acida* extracts and 1, 3-diaminopropane for their antitrypanosomal activities: *In vitro* model

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Using an *in vitro* experimental model, 1,3-diaminopropane and water, and methanol and dichloromethane extracts of *Vernonia amygdalina* (leaf) and *Hymenocardia acida* (leaf, stem bark, root bark) were screened for antitrypanosomal activity at concentrations of 0.25-4 mg/ml. Decrease or arrest of parasites motility coupled with loss of infectivity to mice was taken as the indicator of *in vitro* activity. The results obtained showed that only methanol extract of *V. amygdalina* leaf was active as it arrested parasites motility within 75 min of incubation with minimum inhibitory concentration of 444 µg/ml, and also caused loss of infectivity of the parasites to mice. Dichloromethane extract of *H. acida* root bark and 1, 3-diaminopropane slightly reduced parasites motility, while all other extracts neither reduced the motility nor caused loss of infectivity. These results suggest that the methanol extract of *V. amygdalina* leaf possesses *in vitro* antitrypanosomal activity.

Key words: Antitrypanosomal activity, Infectivity, *Vernonia amygdalina*, *Hymenocardia acida*, 1, 3-diaminopropane dihydrochloride.

INTRODUCTION

African trypanosomiasis, otherwise known as sleeping sickness in humans and '*Nagana*' in cattle, is a disease that is resurgent in Africa. The causative agents are trypanosomes, protozoans belonging to the order *Kinetoplastida*, and family *Trypanosomatidae.* Current chemotherapy of the disease is far from being ideal. The few registered trypanocides are frequently toxic, require lengthy parenteral administration, lack efficacy and are unaffordable for most of the patients (Kieser et al., 2001; Legros et al., 2002). Therefore, there is an urgent need to search for new, safe, and effective molecules that can be developed to new trypanocides.

New drug development is an extremely expensive process, and no pharmaceutical company is interested in spending millions of Dollars in the investigation of a new treatment for a disease which only exists in Africa. One of the remote possibilities is the development or trial of compounds that are active against disease prevalent in the developed countries and which could also be useful for African trypanosomiasis. Medicinal plants and some anticancer agents could well provide lead compounds that in the near future can be developed to anti-trypanosomal agents that are effective, less toxic and affordable. This is encouraged by the fact that several well-known drugs, such as quinine and artemisinin used as antiprotozoal agents, have their origins in nature (Kirby, 1996; Camacho et al., 2000; Tagboto and

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Townson, 2001). Similarly, anticancer agents, particularly those that inhibits polyamine biosynthesis, such as 1,3-diaminopropane, could possess potential antitrypanosomal activity. Therefore, the present study investigates the *in vitro* antitrypanosomal activity of 1,3-diaminopropane dihydrochloride (a synthetic anticancer agent) and crude extracts of *Vernonia amygdalina* and *Hymenocardia acida* plants commonly used in Northern Nigeria for the treatment of various protozoan diseases. Also, their effects on the infectivity of the parasites to mice will be investigated.

MATERIALS AND METHODS

Chemicals/reagents

All chemicals and reagents are of analar grade and were purchased from Sigma-Aldrich Ltd. Different concentrations of 1,3-diaminopropane dihydrochloride were constituted in sterile distilled water.

Plant materials

Candidate plants were selected on an ethnopharmacological basis with assistance of a traditional herbalist. *V. amygdalina* leaf (V. N0 675) was collected from the garden of Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Plateau State. *H. acida* (leaf, stem bark and root bark) (V. N0 900719) was collected from the Dam of Ahmadu Bello University (ABU), Zaria. Identification was done at the herbarium of the Department of Biological Sciences, ABU, Zaria. All plant parts were collected in February.

Preparation of crude plant extracts

All plant parts to be screened were allowed to dry under shed. About 40 g of each dried and powdered part was extracted by reflux with 400 ml of methanol and dichloromethane separately for 3 h. Aqueous extract was prepared by soaking 40 g of dried parts in distilled water and gently heating at 50°C for 3 h. The twelve liquid extracts obtained were then filtered with filter paper and concentrated in a rotary evaporator and then gently and carefully open-dried over water bath at 40°C. The extracts were then kept in a refrigerator at 4°C until required. All extract solutions were reconstituted in phosphate buffered–saline (PBS) or PBS in 10% dimethylsulphoxide (for methanol extracts only) just before use.

Trypanosome parasite

Trypanosoma brucei brucei (Federe strain) was obtained from the Parasitology section of NITR, Vom. The parasites were maintained by serial passage in donor mice. The strain was originally isolated from a goat in Federe Village, Kaduna State, Nigeria, in 1983.

Animals

Male and female albino mice (30-35 g) for the infectivity test were purchased from the small animal house of National Veterinary Research Institute (NVRI), Vom, Plateau State. They were fed on standard animal feeds (Vital feeds, Nig Ltd, Jos) and water *ad libitum.*

In vitro antitrypanosomal activity and infectivity tests

A simple and modified technique described by Kaminsky and Zweygarth (1989a), and Kaminsky and Brun (1993) was used to assay the *in vitro* antitrypanosomal activity of the extracts. Briefly, 10 mg of each extract was dissolved in PBS or PBS in 10% dimethylsulphoxide (methanol extracts) to obtain 20 mg/ml stock solutions. By serial dilutions, different extracts concentrations of 0.25-4 mg/ml were then prepared from the stock solutions. Aliquots of 100 µl of each extract at concentrations of 0.25, 0.5, 1, 2 and 4 mg/ml was then incubated with 200 µl parasitized-saline containing 10^6 *T. b. brucei* parasites and 150 µl phosphate-glucose buffer, in a 96 well titer plate for 2 h at room temperature. This gives effective extract concentrations of 55, 111, 222, 444 and 888 µg/ml (0.055, 0.111, 0.222, 0.444 and 0.888, mg/ml), respectively. Level of parasites motility was checked under microscope and graded every 15 min. The minimum inhibitory concentration (MIC), which is the concentration at which no parasite with motility was seen, was determined microscopically. All tests were done in duplicate.

Infectivity test

In the second stage, after 2 h incubation, each remaining incubation mixture from each well of the plate was then inoculated into two healthy mice to determine if the parasites have lost infectivity or not. Parasitaemia was checked daily for more than 60 days according to the method of Herbert and Lumsden (1976).

RESULTS

The results from *in vitro* test showed that only methanol extract of *V. amygdalina* leaf caused arrest of parasites motility as the parasites were completely immobilized within 75 min of incubation with MIC 444 µg/ml (Figure 1). Figures 2 and 3 shows that dichloromethane extract of *H. acida* root bark and 1,3-diaminopropane only reduced the parasites motility but did not cause complete immobility within 90 and 120 min of incubation, respectively. While all other extracts caused no change in the parasites motility compared to the control (figures not shown).

Results on infectivity test shows that all mice inoculated with remaining incubation mixtures developed infection and died within 11 days post-inoculation (pi) except those in which the inoculums contained methanol extract of *V. amygdalina* leaf. This set of mice survived more than 60 days without parasites detected in their blood circulation (Figure 4). Figures 5 and 6 shows that dichloromethane extract of *H. acida* root bark and 1,3-diaminopropane did not cause loss of infectivity of the parasites to the mice because the mice developed infection and eventually died (day 8-11 pi and day 9-11 pi, respectively) as did the controls (day 6).

DISCUSSION

Among the twelve extracts screened for *in vitro* antitrypanosomal activity, only the methanol extract of *V*

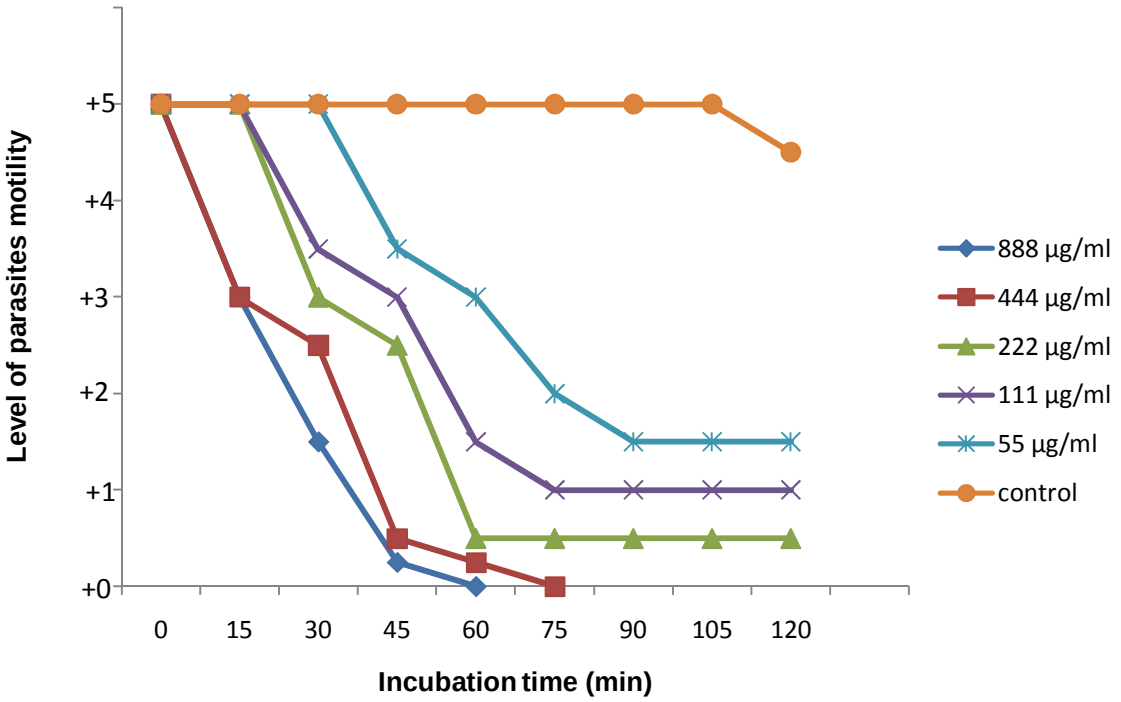

Figure 1. The *in vitro* antitrypanosomal activity of methanol extract of *Vernonia amygdalina* leaf against *Trypanosoma brucei brucei.* Key: +0 = No motility; +1 = lethargic; +2 = Slight; +3 = Moderate; +4 = Active; +5 = Very active.

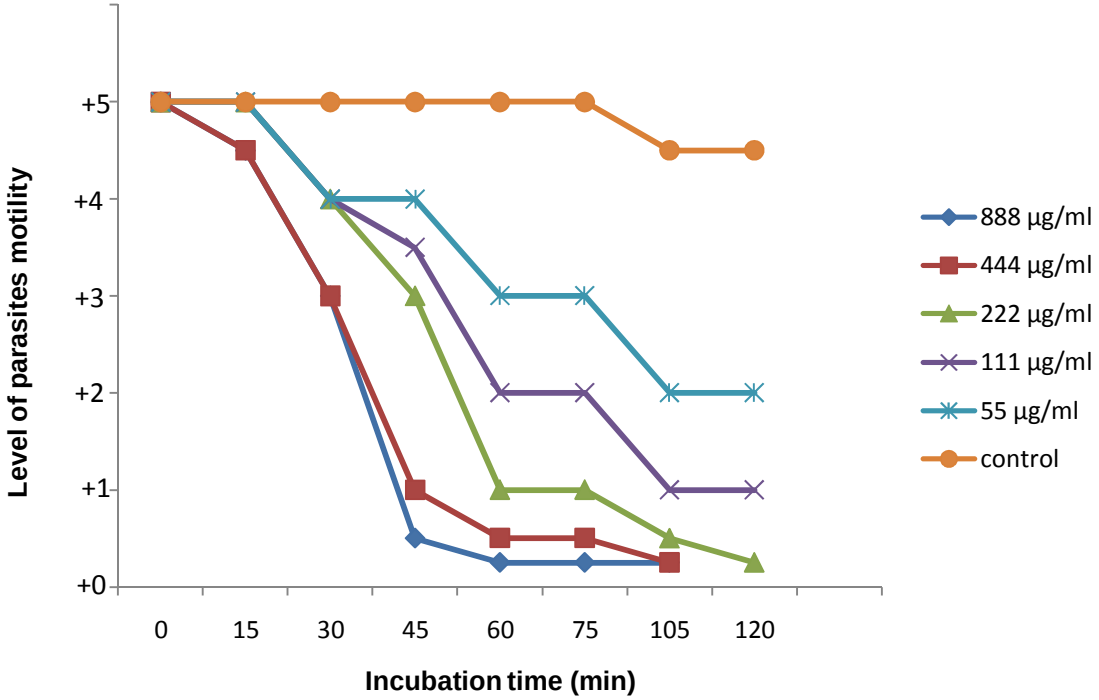

Figure 2. The *in vitro* antitrypanosomal activity of dichloromethane extract of *Hymenocardia acida* root bark against *Trypanosoma brucei brucei.* Key: +0 = No motility; +1 = lethargic; +2 = Slight; +3 = Moderate; +4 = Active; +5 = Very active.

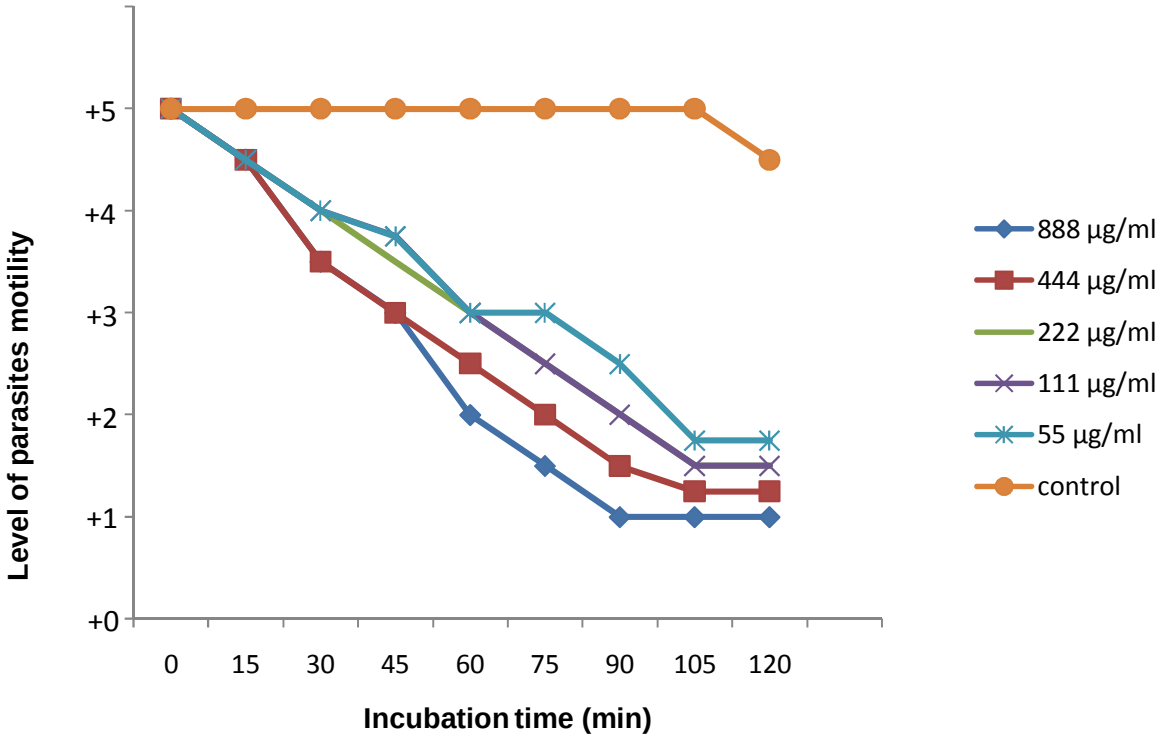

Figure 3. The *in vitro* antitrypanosomal activity of 1,3-diaminopropane against *Trypanosoma brucei brucei.* Key: +0 = No motility; +1 = lethargic; +2 = Slight; +3 = Moderate; +4 = Active; +5 = Very active.

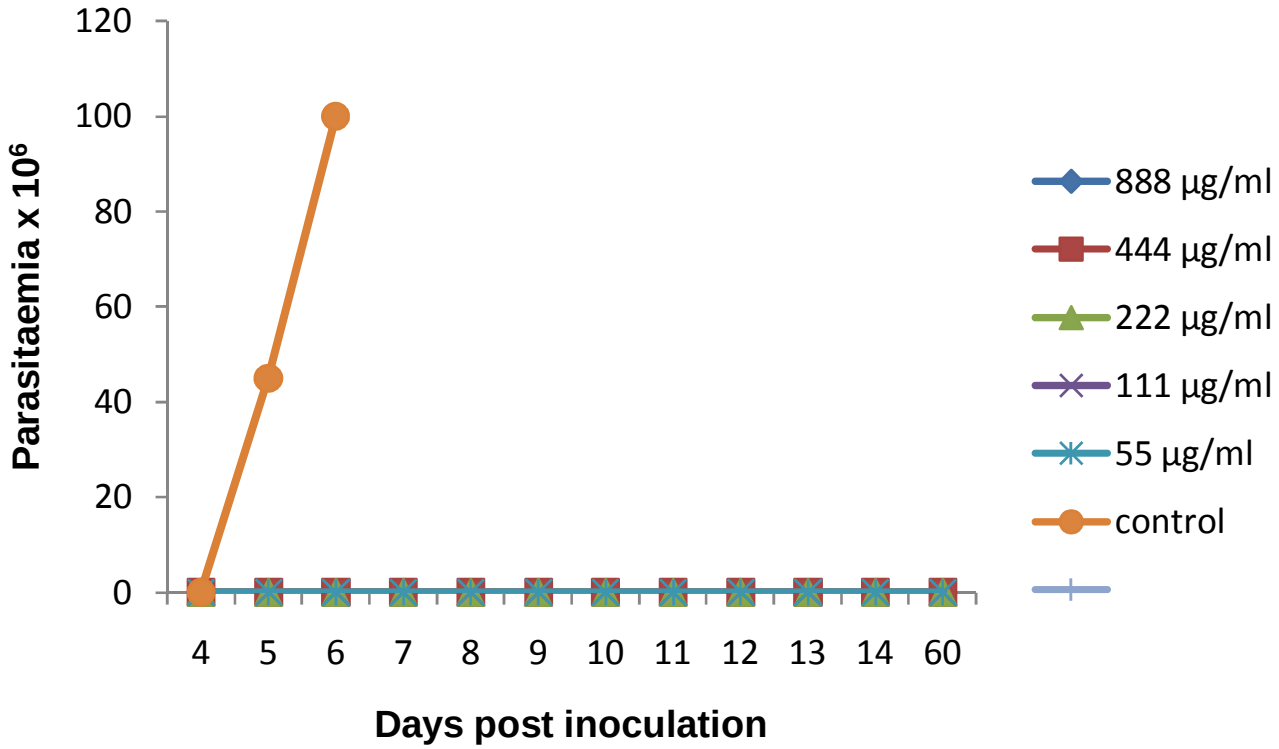

Figure 4. Effect of methanol extract of *Vernonia amygdalina* leaf on infectivity of *Trypanosoma brucei brucei* to mice.

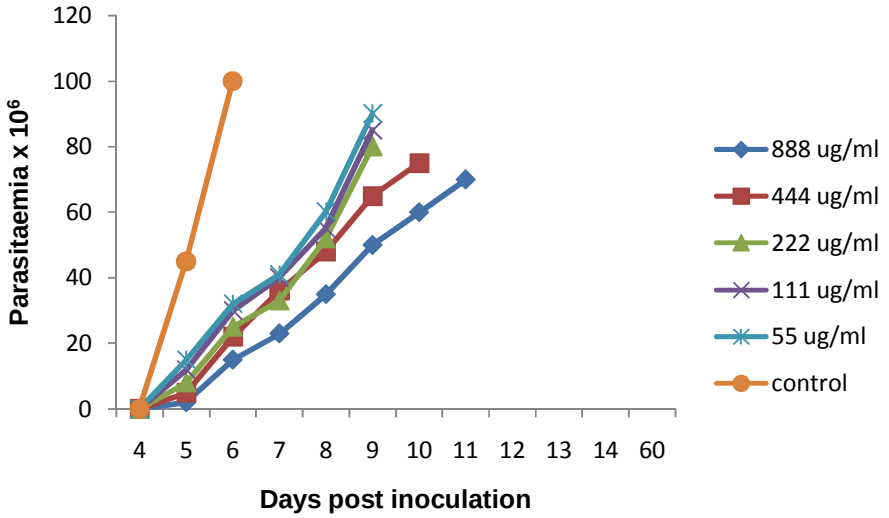

Figure 5. Effect of dichloromethane extract of *Hymenocardia acida* root bark on infectivity of *Trypanosoma brucei brucei* to mice.

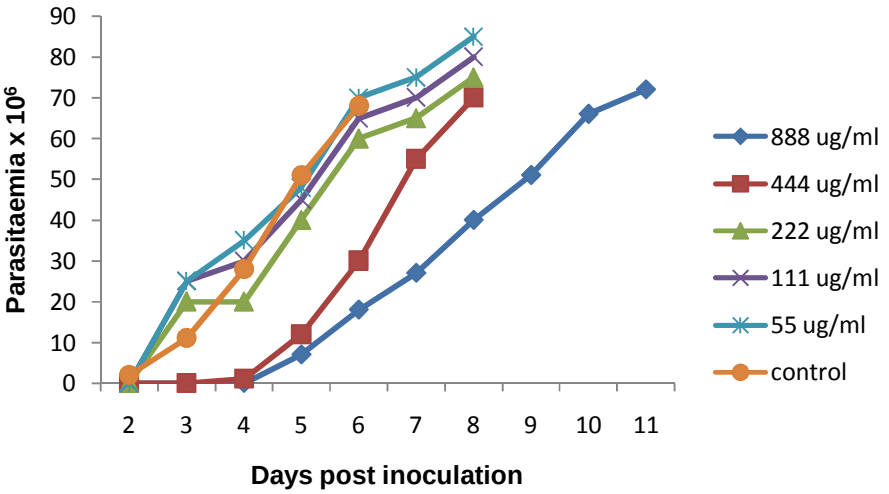

Figure 6. Effect of 1,3-diaminopropane on infectivity of *Trypanosoma brucei brucei* to mice.

. amygdalina leaf possessed activity against *T. b. brucei* as it completely immobilized the parasites within 75 min of incubation with MIC 444 µg/ml. Parasites motility constitute a relatively reliable indicator of viability of most zoo flagellate parasites (Peter et al., 1976; Kaminsky et al., 1996) and arrest or drop in motility of trypanosomes may serve as a measure of antitrypanosomal activity of crude plant extracts when compared to the control, phosphate-glucose buffer saline. Similarly, Atawodi et al. (2003) reported that complete elimination of motility or reduction in motility of parasites when compared to the control could be taken as indices of trypanocidal activity.

However, it should be stated here that in this *in vitro* model, complete immobility of the parasites does not necessarily indicated that the parasites were dead, but rather the parasites may have lost their infectivity. This was confirmed through the infectivity test which showed that this extract inhibited healthy mice from developing infection for more than 60 days unlike the other extracts and the control that died within day 6-11 pi. This suggests that the methanol extract of *V. amygdalina* leaf may have caused loss of infectivity by abrogating some vital metabolic processes in the parasites within 75 min of incubation. Interestingly, even though the extract at the concentrations of 55, 111 and 222 µg/ml did not cause complete immobility of the parasites, yet it caused loss of infectivity of the parasites to mice. Perhaps, this could be due to some morphological changes on the parasites

induced by the extract at these concentrations that render them more susceptible to the mice immune defense systems.

Both *V. amygdalina* and *H. acida* have been investigated in other studies for antitrypanosomal activities. Wurochekke and Nok (2004) reported that aqueous extract of *V. amygdalina* leaf did not have *in vitro* antitrypanosomal activity. This is in agreement with the findings in our present study. However, contrary to our findings, other studies (Hoet et al., 2004; Freiburghaus et al., 1996) have reported methylene chloride and petroleum ether extracts of *H. acida* leaf and root bark, respectively, to have strong *in vitro* activity. The differences in their findings and the present study may be due to different solvents of extraction used, known variations in the chemical composition of plants according to geographical area and the time and season of collection.

The anticancer agent, 1,3-diaminopropane is an inhibitor of ornithine decarboxylase (ODC), similar to diflouromethylornithine (DFMO), the only antitrypanosomal agent introduced in last 40 years (Pegg, 1988). ODC is the first enzyme in the biosynthesis of polyamines, and together with the other enzymes in the pathway, have been the major targets for the development of new trypanocidal agents (Brun et al., 1996; Cyrus et al., 1996; Sylke et al., 2001). In the present study, DAP did not show activity *in vitro* against *T. b. brucei* but it is recommended that it should be re-try both *in vitro* and *in vivo* and so also other polyamine inhibitors, because these compounds have the potentials to be developed as lead compounds for new trypanocidal agents.

Conclusion

Overall, results from this work have encouraged us to carry out more investigations to isolate the active principles and elucidate their mechanism of actions in order to fully harness the antitrypanosomal potential of methanol extract of *V. amygdalina* leaf.

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