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Experimental chemotherapy with *Allium sativum* (Liliaceae) methanolic extract in rodents infected with *Leishmania major* and *Leishmania donovani*

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Abstract

Background & objectives: Several plant products have been tested and found to possess antileishmanial activity. The present study was undertaken to establish whether methanolic extract of *Allium sativum* Linn has antileishmanial activity in comparison to standard drugs.

Methods: Methanolic extract of *A. sativum* bulbs was screened for *in vitro* and *in vivo* antileishmanial activity against *Leishmania major* strain (NLB 145) and *L. donovani* strain (NLB 065). Pentostam® and Amphotericin B® were used as standard drugs. BALB/c mice and golden hamsters (*Mesocricetus auratus*) were used in *in vivo* studies on *L. major* and *L. donovani* respectively.

Results: The extract exhibited very low cytotoxicity (IC_{50} >450 µg/ml) against Vero cells. The extract had significantly better (p <0.001) leishmanicidal activity against both species (IC_{50} 34.22 µg/ml to *L. major*, 37.41 µg/ml to *L. donovani*) than Pentostam. However, the activity was significantly lower (p <0.001) than that of Amphotericin B against both the species. At a concentration of 250 µg/ml, the extract induced the production of 60 µM of nitric oxide, a ten-fold up-regulation in activated macrophages. The multiplication indices for *L. major* amastigotes treated in 100 µg/ml were significantly different (p <0.05). Treatment with the extract, daily for 28 days led to a significant reduction (p <0.05) in footpad swelling in BALB/c mice; similar activity noticed in the treatment with standard drugs. The Leishman-Donovan Units (LDU) for the extract treated animals were significantly higher (p <0.05) than those of standard drugs, but lower compared to the negative control.

Interpretation & conclusion: Since the mechanism of action for the methanolic extract is apparently immunomodulatory, garlic compounds could be purified and tried as complementary medicine in the management of leishmaniases.

Key words Amastigotes and promastigotes; antimonials; leishmaniasis; methanolic extract

Introduction

Leishmaniases are diseases caused by obligate intracellular, kinetoplastid protozoa of the genus *Leishmania* and family Trypanosomatidae[1]. The diseases caused by *Leishmania* continue to have a major impact on much of the world's population and are currently considered to be the emerging illnesses with high morbidity and mortality in the tropics and subtropics[2,3]. Urban development, destruction of forests, environmental changes, human migrations to areas where the disease is endemic and wars have

contributed to an increase in the incidence of leishmaniases[4,5].

In the absence of a vaccine, the global burden of leishmaniases has remained stable for some years, causing a morbidity and mortality loss of 2.4 million disability adjusted life-years (DALYs) and approximately 70,000 deaths, a significantly high rank among communicable diseases[6,7]. Currently, the drug of choice in the treatment of lesion caused by *L. major* in Kenya is the pentavalent antimony, sodium stibogluconate (Pentostam®). Sodium stibogluconate and other antimonials though effective, are expensive, toxic and require a protracted administration in patients[8].

Several plant products have been tested and found to possess some antileishmanial activity. *Allium sativum*, commonly known as garlic, is a species of the onion family (Alliaceae). A bulb of garlic, the most commonly used part of the plant divided into numerous fleshy sections called cloves, which are used as seeds for consumption and for medicinal purposes. *Allium sativum* has been found to be a powerful rubefacient, antitusive, diaphoretic and vermifuge agent[9,10]. The present study sought to establish whether methanolic extract of *A. sativum* has immunotherapeutic activity against *L. major* and *L. donovani in vitro* and *in vivo* in comparison to the standard drugs, Pentostam® and Amphotericin B®.

Material & Methods

Allium sativum extract preparation: The extract was prepared according to the method of Mantis *et al*[11]. Briefly, bulbs were peeled, chopped into small pieces and air-dried until constant weight then ground with an electric blender. Methanolic extraction was done using absolute methanol as the organic solvent. The final dry extract was used to make the solution for injection and oral treatment.

Cultivation of Leishmania promastigotes: These cultures of *L. major* and *L. donovani* were maintained in BALB/c mice and golden hamsters, respectively, by serial subcutaneous passage to maintain their virulence[12]. An aspirate from a footpad of an infected BALB/c mouse or a splenic biopsy from the hamster was grown in Schneider's *Drosophila* insect medium (SIM-F), supplemented with 20% heat inactivated foetal bovine serum (FBS), 500 µg/ml penicillin, 500 µg/ml streptomycin and 250 µg/ml of fluorocystosin arabinoside[13,14]. Stationary-phase promastigotes were harvested by centrifugation at 1500 g for 15 min at 4°C. The pellets were washed thrice in sterile PBS by centrifugation.

Cytotoxicity assay: Vero cells were cultured and maintained in minimum essential medium (MEM) supplemented with 10% FBS. The cells were cultured at 37°C in 5% CO_2, harvested by trypsinization, pooled in a 50 ml vial and 100 µl cell suspension, used to test toxicity by adding 150 µl of the highest concentration of the extract and serial diluting. The controls used were cells with no extract and medium alone. MTT reagent (10 µl) was added, after 4 h it was aspirated off, and then 100 µl of dimethylsulfoxide (DMSO) was added and the plates were shaken for 5 min. The absorbance was measured for each well at 562 nm using a micro-titer plate reader[15].

Evaluation of minimum inhibitory concentration (MIC): Leishmania promastigotes (1×10^6 parasites/ml) were maintained in culture or in the presence of several concentrations of the *A. sativum* extract. Cell growth was evaluated daily by assessment of visibility and turbidity in order to evaluate the minimum inhibitory concentration (MIC).

Anti-promastigote assay: Promastigotes were incubated in 24-well plates in the presence of different concentrations of the extract for five days. Aliquots of parasites were then transferred to 96-well microtiter plate, incubated at 27°C in 5% CO_2 for 24 h, then 200 µl of highest concentration of the extract was added and serially diluted, then incubated further at 27°C for 48 h. The controls used were promastigotes with no extract and medium alone. MTT reagent (10 µl) was added and incubated for

4 h, then the medium together with MTT was aspirated off; DMSO (100 µl) was added and the plates were shaken for 5 min. The absorbance was measured for each well at 562 nm using a micro-titer plate reader[16].

Antiamastigote assay: This was carried out as described by Delorenzi *et al*[17]. Treatment of infected macrophages with the extract was done once. Pentostam and amphotericin were used as positive controls for parasite growth inhibition while PBS was used as a negative control. The medium and extract 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 stain. The number of amastigotes was determined by counting at least 400 macrophages in duplicate cultures, and the results expressed as infection ratio (IR), and multiplication index (MI)[18].

$$\text{IR} = \text{No. of infected macrophages in 100 macrophages}$$

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

Nitric oxide production determination: Nitric oxide release in supernatants of macrophage culture was measured by the Griess reaction for nitrites[19]. The supernatants were collected (100 µl) 48 h after introducing the extract into the culture medium. The assay was done in triplicate wells in a 96-well microtiter plate. To this, 60 µl of Griess reagent A (1% sulphanilamide in 1.2 M HCl) and then 60 µl of Griess reagent B (0.3% N-[1-naphthyl]ethylenediamine) was added. The plates were read at 540 nm in an ELISA plate reader.

Infection and treatment of BALB/c mice and golden hamsters: The left hind footpads of BALB/c mice were inoculated with 1×10^6 stationary phase culture of *L. major* promastigotes in 40 µl phosphate buffered saline (PBS) intradermally. Lesion development was monitored by measuring the thickness of the infected footpad using a Vernier caliper weekly. Treatment with the extract started one month post-infection, and groups of mice were treated for four weeks by oral administration using a canula or by intraperitoneal injections of 20 mg/kg/day of the extract. To compare the drug effects, the lesion size was measured and expressed as the difference in thickness between the infected and the uninfected contralateral footpad. The hamsters were inoculated with 2×10^6 stationary phase culture of *L. donovani* promastigotes in 40 µl PBS intraperitoneally. Treatment with the extract started one month post infection and groups of hamsters were treated for four weeks by oral administration using a canula or by intraperitoneal injections of 20 mg/kg/day of the extract.

Quantifying parasite burden from spleens: Impression smears of spleen of treated animals were made as described by Chulay and Bryceson[20]. The slides were examined under a compound microscope for enumerating the number of amastigotes per 1000 host nuclei. The relative and total numbers of parasites in the spleen, named Leishman-Donovan Units (LDU) and the total Leishman-Donovan Units (total LDU) respectively were calculated according to the formula by Bradley and Kirkley[21].

$$\text{LDU} = \text{No. of parasites/1000 host nuclei}$$

$$\text{Total LDU} = \text{LDU} \times \text{organ weight} \times 2 \times 10^5$$

Statistical analyses: Data were presented as mean and standard error mean or standard deviation. Comparison between multiple groups was performed by analysis of variance (ANOVA) and when significant, comparisons between two groups were performed by student's *t*-test. All analyses were carried out at 5% level of significance.

Results

In vitro activities of A. sativum extract against L. major and L. donovani promastigotes: The extract was found to have low toxicity (IC_{50} >450 µg/ml) against Vero cells. The extract showed IC_{50} values

Table 1. The *in vitro* activities of *A. sativum* extract against *L. major* and *L. donovani* promastigotes

Treatment	*L. major*		*L. donovani*	
	IC_{50} ((µg/ml)	MIC (µg/ml)	IC_{50} (µg/ml)	MIC (µg/ml)
A. sativum extract	34.22	250	37.41	250
Amphotericin B	1.74	62.5	1.18	250
Pentostam	127.77	250	163.83	500

of 34.22 µg/ml and 37.41 µg/ml against *L. major* and *L. donovani* promastigotes respectively, compared to 1.74 µg/ml against *L. major* and 1.18 µg/ml against *L. donovani* for Amphotericin B (Table 1).

In vitro activities of A. sativum extract against L. major and L. donovani amastigotes: At a concentration of 100 µg/ml of the extract, the infection rates were 13 and 15% for *L. major* and *L. donovani* amastigotes respectively. At this concentration, the infection rates in Amphotericin B treated macrophages were 9% for *L. major* and 11% for *L. donovani* amastigotes. Pentostam treated macrophages showed infection rates of 10 and 11% against *L. major* and *L. donovani* amastigotes respectively (Table 2). The difference in infection rates between the extract and the standard drugs treated *L. donovani* and *L. major* amastigotes was not statistically significant ($p > 0.05$).

Table 2. The *in vitro* activities of *A. sativum* extract against *L. major* and *L. donovani* amastigotes. Data represent percent infection rates

Treatment	Extract concentration (µg/ml)	Infection rate (%)	
		L. major	*L. donovani*
A. sativum extract	100	13	15
	50	15	17
	25	22	21
Amphotericin B	100	9	11
	50	15	13
	25	18	15
Pentostam	100	10	11
	50	14	15
	25	18	18
RPMI	–	52	55

The multiplication indices for *L. major* and *L. donovani* amastigotes in macrophages treated with 100 µg/ml of the extract were significantly different ($p < 0.05$) (Fig. 1). At a concentration of 25 µg/ml, *L. major* amastigotes showed multiplication indices of 67.87% in the extract, 42.86% in Amphotericin B

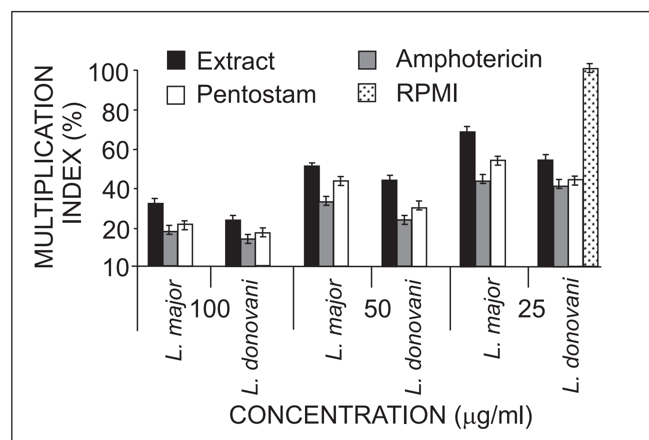

Fig. 1: The *in vitro* activities of *A. sativum* extract against *L. major* and *L. donovani* amastigotes. Data represent mean ± S.E. of the multiplication indices of experiments done in triplicate.

and 53.57% in Pentostam. These indices were significantly different ($p < 0.001$).

Stimulation of nitric oxide production: The extract triggered production of 26 µM of nitric oxide at a concentration of 31.25 µg/ml compared to 6 µM for the untreated macrophages. At a concentration of 250 µg/ml, the extract induced production of 60 µM of NO; a ten-fold up-regulation of nitric oxide production compared to the negative control (Table 3).

Table 3. Nitric oxide produced (μM) by macrophages treated with different concentrations of *A. sativum* extract

Conc. (μg/ml)	*A. sativum* extract NO conc. (μM)	RPMI NO conc. (μM)
31.25	26	6
62.5	30	–
125	32	–
250	60	–

Effects of A. sativum extract on body weight and lesion size progression in L. major-infected mice: At the end of the study, the difference in average body weights of animals treated orally and intraperitoneally were not statistically significant ($p > 0.05$). The healing effect of the extract and standard drugs on lesion size was noticeable after seven days of treatment (Figs. 2a and b). At the end of the treatment period, the mean lesion sizes in all the treatment groups were significantly lower compared to the negative control ($p < 0.05$).

Effects of A. sativum extract on the parasite burden in L. donovani-infected hamsters and L. major-infected mice: The parasite loads for hamsters treated orally and intraperitoneally with the extract were not significantly different ($p > 0.05$). However, there was a significant difference in parasite numbers recovered from Amphotericin B treated hamsters and extract treated hamsters for oral and intraperitoneal administration respectively ($p < 0.05$). The difference in parasite burdens of hamsters treated with the extract and the PBS was highly significant ($p = 0.0001$) (Fig. 3a).

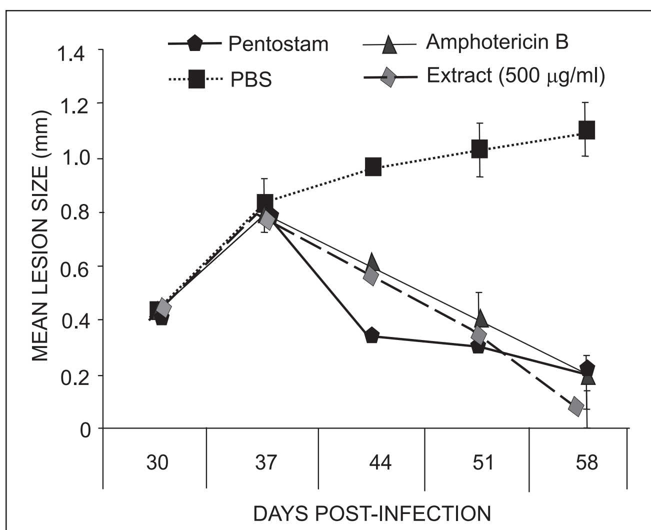

Fig. 2a: The effect of oral treatment of *L. major*-infected mice with *A. sativum* extract on the progression of footpad lesions. Data represent mean ± S.E. derived from three mice per group.

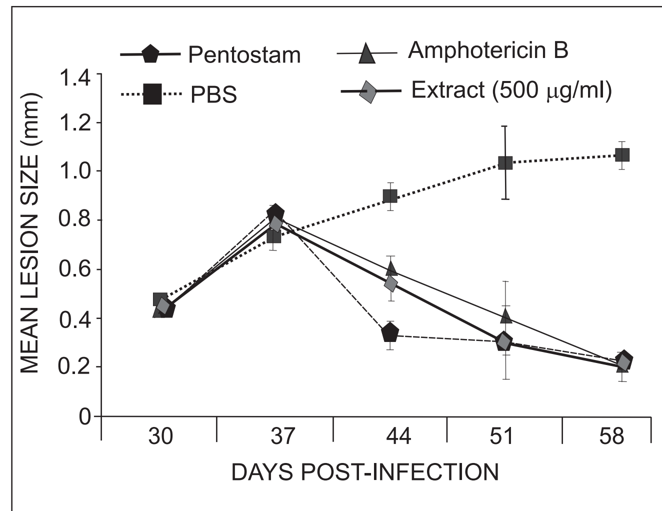

Fig. 2b: The effect of intraperitoneal treatment of *L. major*-infected mice with *A. sativum* extract on the progression of footpad lesions. Data represent mean ± S.E. derived from three mice per group.

The LDU values of BALB/c mice treated with the extract were significantly higher than that of Amphotericin B and Pentostam ($p < 0.05$). Compared to the PBS treated group, the total LDU values for the extract treated groups were significantly lower ($p < 0.001$) (Fig. 3b).

Discussion

Previous studies using medicinal plant extracts in the control of leishmaniases have demonstrated the effectiveness of these natural products as alternative therapies[22, 23]. *In vitro* studies have shown antileishmanial activities in a range of plants including *Waburgia ugandensis*[24]; *M. senegalensis*[1] and *Aloe vera*[25]. A study by Gamboa-Leon *et al*[26] demonstrated that an aqueous extract of dried garlic could

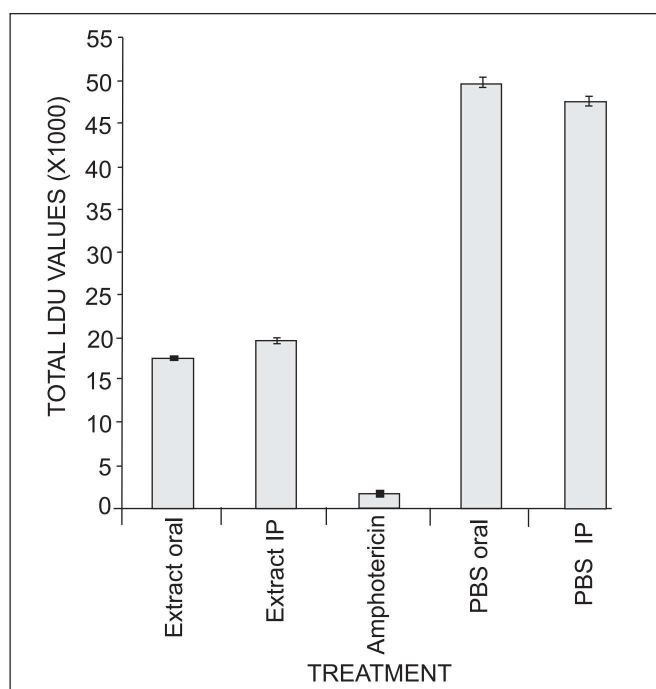

Fig. 3a: Parasite burden in spleens of *L. donovani*-infected golden hamsters 6 days after the end of treatment. Data presented as mean ± S.E. of number of amastigotes per 1000 host nuclei.

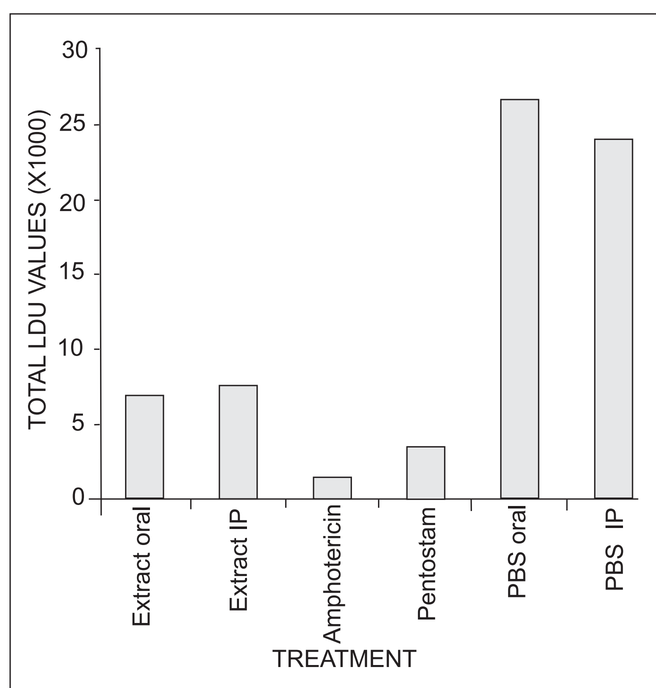

Fig. 3b: Parasite burden in spleens of *L. major*-infected BALB/c 6 days after the end of treatment. Data presented as mean ± S.E.M. of number of amastigotes per 1000 host nuclei.

control infection by *L. mexicana* in BALB/c mice. In this study, these previous findings are extended to demonstrate that dried garlic methanolic extract could control infections by *L. major* in BALB/c mice and *L. donovani* in golden hamsters and that these chemotherapeutic effects compare well with the standard drugs *in vivo*. The findings in this study further demonstrate that unlike most of the standard antileishmanial drugs, which require protracted parenteral therapy and are toxic, an agent derived from garlic can be administered either orally or intraperitoneally with similar effectiveness against *Leishmania* parasites.

In vitro tests in this study demonstrated that the activities of *A. sativum* extract against *L. major* and *L. donovani* promastigotes are significantly better than those of the standard drug Pentostam; however, Amphotericin B had better leishmanicidal activity than the extract. These findings underscore the potential of *A. sativum* derived agents in the control of both visceral and cutaneous leishmaniases.

The results from this study show an increase in the number of amastigotes in macrophages with a decrease in concentration for the extract and the standard drugs. The infection rates for both *L. major* and *L. donovani* were higher in *A. sativum* extract treated macrophages than in the positive controls. Likewise, the number of amastigotes in macrophages treated with the extract compared to positive controls at 100 μg/ml were significantly higher. However, the infection rates were higher in RPMI treated macrophages than in *A. sativum* extract treated macrophages by about ten-fold.

The results obtained from this study indicate that *A. sativum* extract induce production of nitric oxide in activated macrophages, which serves as a defence mechanism against *Leishmania* amastigotes. Hollzmuller *et al*[19], demonstrated that natural resistance to *Leishmania* infection depends on intracellular parasite killing by activated macrophages through production of L-arginine-nitric oxide metabolic pathway.

The findings in this study on the progression of disease by measuring lesion size in the course of treatment indicated that therapeutic effects of *A. sativum* extract administered either orally or intraperitoneally compare well with the standard drugs that require parenteral administration only. The time taken for the extract to take effect, demonstrated by a significant reduction in lesion size, was the same (14 days of treatment) compared to the standard drugs. After 21 days of treatment, there was no difference in the lesion sizes of treated animals, i.e. the healing time is similar. Apparently, all the treatment drugs were well-tolerated by the animals as the body weights were not significantly different at termination. As shown earlier by *in vitro* tests, the standard drugs and *A. sativum* extract could be having different parasite killing mechanisms but their efficacy is the same. The LDU values obtained in this study indicate the route of administration of the extract does not have a significant effect on its efficacy. Compared to the negative control, *A. sativum* extract reduced parasitaemia in both mice and hamsters significantly.

Conclusion

Allium sativum extract has a high potential of being used in the treatment of leishmaniases. This study has demonstrated that *A. sativum* preparations can be administered orally. Being a natural product, the extract may be safe to use; with reduced toxicity compared to the standard drugs. Further studies need to be carried out using non-human primates such as vervet monkeys (*Cercopithecus aethiops*) to confirm whether the extract can be as effective in these primates for future use to treat the leishmaniases. Since the mechanism of action for garlic extract in killing *Leishmania* parasites is apparently immunomodulatory, the garlic compounds could be examined as complementary medicine in the management of leishmaniases. The emergence of antimonial-resistant *Leishmania* strains is on the rise and natural products like *A. sativum* extracts and other plant products that have been tested and found to possess antileishmanial activities may provide alternative treatment.

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