Toxicity and efficacy of aqueous crude extracts from *Allium sativum*, *Callistemon citrinus* and *Moringa stenopetala* against *L. Major*

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Abstract
Cutaneous leishmaniasis (CL) treatment involves pentavalent antimonials, amphotericin B, pentamidine, miltefosine among others. These drugs are toxic, costly, and require prolonged use. CL is a protozoan skin infection which may lead to disfiguring and stigmatization. In Kenya, CL is common in Baringo County where it is caused by *Leishmania major* and transmitted by infected female phlebotomine sand fly. Leishmaniases are common in poverty stricken areas where victims opt for local herbal therapies. Herbs used haven't been tested scientifically to verify their toxicity and efficacy. The current study determines *in vitro* toxicity and *in vivo* efficacy of aqueous crude extracts of *Moringa stenopetala*, *Callistemon citrinus*, and *Allium sativum* against *L. major*. The IC_{50} of aqueous extracts against promastigotes ranged from 297μg/ml to 575μg/ml compared to Pentostam and liposomal amphotericin B with IC_{50} of 0.26μg/ml and 0.82μg/ml respectively. The viability of promastigotes upon exposure to extracts ranged from 52.55% to 60.57%. Similarly the IC_{50} of extracts against vero cells ranged between 467μg/ml to 2105μg/ml compared to 108μg/ml and 60μg/ml for pentostam and liposomal amphotericin B respectively. Orally administered *A. sativum* reduced *L. major* caused footpad lesions significantly ($P < 0.05$) when compared to control PBS. The efficacy of oral *C. citrinus* extracts (B) in reducing amastigotes in spleens of infected BALB/c mice was 82.99%, followed by oral *M. stenopetala* (A) at 66.96% and oral *A. sativum* (C) at 60.37% compared to pentostam and liposomal amphotericin B at 66.40% and 60.62% respectively. The difference between the mean total LDUs for aqueous oral *C. citrinus* extracts and control oral PBS was significant ($P = 0.017$). It was concluded that crude aqueous extracts of *A. sativum*, *M. stenopetala*, and *C. citrinus* show antileishmanial activity at low toxicity. Inclusion of garlic and moringa in the diets of people in leishmaniases foci should be emphasized.

Key words:M. stenopetala, *C. citrinus*, *A. sativum*, efficacy, toxicity, aqueous extracts, *Leishmania major*, vero cells

Introduction
The Leishmaniases are sandfly-transmitted protozoal tropical diseases prevalent in more than 80 countries in the world. Approximately 12 million people in 88 countries are infected with leishmaniases and 2 million new cases occur each year (Anez *et al*., 1999). Leishmaniases are infectious diseases caused by parasites of the genus *Leishmania*. Cutaneous leishmaniasis (CL) which causes skin ulcers at the site of the bite by an infected sand fly, has an estimated incidence ranging from 0.7 to 1.2 million cases each year according to Alvar *et al*. (2012). In Kenya, CL is prevalent in Baringo County in the Rift Valley region

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where the causative agent is *Leishmania major* (Gicheru *et al*., 2001). Although CL is less fatal, it causes severe pain, stigmatization, disfiguring when the lesions are accumulated and it requires prolonged treatment with expensive and toxic drugs among them being pentavalent antimonials and amphotericin B.

Cutaneous leishmaniases tend to occur in poverty stricken and war torn areas of the world (Clem, 2010) where the affordability of the standard conventional drugs by the patients may be a challenge (den Boer *et al*., 2011). Many patients therefore seek for herbal therapies which are cheaper and readily available. However, most of the herbal materials have not been evaluated scientifically for their efficacy and toxicity. According to Kigondu *et al*. (2009), the efficacy dosage, safety and active principles of most of the herbal preparations are not known. Therefore there is need for an intensive search for local alternative cheaper and safer natural therapies especially in developing countries for the neglected leishmaniases. Herbal products are increasingly becoming important because they symbolize safety in contrast to the synthetics (Joy *et al*., 2001). Use of herbal drugs as combinations has also been in practice for long in many cultural systems to treat various infectious diseases (Gathirwa *et al*., 2008; Yousefi *et al*., 2009). Efficacy of combining herbal materials or drugs will often depend upon the interaction of the individual components in the combination or blend. According to Tahany *et al*., (2010), synergistic and additive interactions are advantageous because the resultant efficacy of the combination is higher.

A. sativum (garlic) is a common perennial tropical plant in the family Amaryllidaceae which is used as food, spice and medicine. Medicinal properties of *A. sativum* are multiple and they range from antimicrobial, hypolipidemic, antithrombotic to antitumour activities (Augusti, 1996). The medicinal properties of garlic have been attributed to organosulfur compounds present in the bulbs (Islam *et al*., 2011). *Moringa stenopetala* Cufodontis is a smooth barked tree in the family Moringaceae and it grows naturally on Lake Baringo islands in Kenya. In southern Ethiopia, the leaves and fruits of *M. stenopetala*, are eaten as vegetables since they are rich in proteins, calcium, phosphorous, iron, and vitamins A and C. The medicinal property of *M. stenopetala* includes treatment of stomach problems, malaria, hypertension, diabetes, asthma and expelling retained placenta (Mekonnen *et al*., 1999). The active components of *M. stenopetala* are mainly glucosinolates (Mekonnen & Drager, 2003). *Callistemon citrinus* is an ornamental evergreen tree in Kenya commonly known as bottle brush tree and it belongs to family Mytaceae. The medicinal property range from antistaphylococcal, nematocidal, larvicidal, pupicidal, antioxidants and antithrombotic (Ali *et al*., 2010) as well as antifungal (Dongmo *et al*., 2010). The genus *Callistemon* is known in folk medicine as anticough, antibronchitis,and insecticidal agent (Abdelhady & Aly, 2012).

It is against this background that the present study was designed to investigate the *in vitro* toxicity of crude aqueous extracts from *A. sativum*, *C. citrinus*, and *M. stenopetala* using *L. major* promastigotes and vero cells and also to establish the *in vivo* efficacy of the crude extracts against *L. major* in BALB/c mice.

Materials and Methods

Plant materials

Young leaves of *M. stenopetala* trees were picked on the slopes of Lake Baringo islands in Kenya while *Callistemon citrinus* flowers were harvested from selected homesteads in Nakuru County, Kenya. Bulbs of *A. sativum* were purchased from Nakumat super market at Nairobi, Kenya. The study plants were positively authenticated at University of Nairobi Herbarium, in the department of Botany, Chiromo Campus. The young leaves of *M. stenopetala*, flowers of *C. citrinus* and thin slices of *A. sativum* cloves were separately dried at room temperature at Kenya Medical Research Institute (KEMRI), *Leishmania* laboratory, until they became brittle and attained a constant weight. The dried plant materials were labeled and then transferred to the Center of Traditional Medicine & Drug Research (CTMDR) at KEMRI, where they were separately ground into powders using an electric mill, labeled and then extracted using distilled water.

Herbal materials Extraction

The aqueous extracts were prepared as described by Delahaye *et al*. (2009). Briefly, 100g of the dried ground plant material in 600 ml of distilled water was placed in a water bath at 70^{o}C for 1.5 hours. The mixture was filtered using Whatman No 1 filter papers and then the filtrate was freeze dried and weighed. The aqueous extracts of *M. stenopetala, C. citrinus,* and *A. sativum* were coded as extracts A, B and C respectively. The extracts were then stored at 4^{o}C until required for the bioassays.

Culturing of *Leishmania* parasites

The *Leishmania major* strain (IDUB/KE/94=NLB-144) used was acquired from Institute of Primate Research (IPR), Kenya where it had been maintained by cryopreservation in liquid nitrogen. The parasites were grown to stationary phase at 25^{o}C in Schneider's insect medium supplemented with 20% heat inactivated fetal bovine serum, 100 U/ml penicillin and 500μg/ml streptomycin (Hendricks & Wright, 1979), and 250μg/ml of 5-fluorocytosine arabinoside (Kimber *et al*., 1981). The stationary-phase metacyclic stage promastigotes were then harvested by centrifugation at 1500 rpm for 15 minutes at 4^{o}C. The metacyclic promastigotes were then used for the *in vitro* and *in vivo* assays.

Culturing of vero cells

The Vero cells were obtained from KEMRI, Nairobi, from the Center for Viral Research (CVR) where they had been cryopreserved in liquid nitrogen to ensure the best quality. The vero cells (100μl) were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS (FBS-HYCLONE® USA), 100U/ml penicillin and 100μg/ml streptomycin in a humidified incubator with 5 % CO_2 in air at 37^{o}C. The vero cells were plated at a concentration of 3×10^5 cells per ml of the culture medium in petri dishes and allowed to grow for 24 hours until a well spread monolayer was formed. The vero cells monolayer was then used for cytotoxicity studies.

Experimental animals

Eight week old inbred BALB/c mice of the same sex were used for *in vivo* efficacy test of the plant extracts. The inbred BALB/c mice were obtained from International Livestock Research Institute (ILRI), Kenya. They were then housed at KEMRI animal house under a temperature range of 23^{o}C to 25^{o}C and were fed on standard commercial diet in the form of mice pencils and were given tap water *ad libitum*. The mice were handled in accordance with the regulations set by Animal Care and Use Committee (ACUC) at KEMRI, Kenya.

Determination of IC_{50} of extracts using *L. major promastiigotes*

This was evaluated as described by Wabwoba *et al*. (2010). The metacyclic promastigotes at a concentration of $1x10^6$ promastigotes per ml were incubated in 24-well plates in presence of different concentrations of plant extracts for five days at 25^{o}C. The aliquots of parasites were then transferred into 96-well micro-titer plates, incubated at 27^{o}C in 5% CO_2 for 24 hours and 200μl of the test *M. stenopetala* (A), *C. citrinus* (B), and *A. sativum* (C) extracts samples were added at concentrations ranging from 5 mg/ml to 0.5 mg/ml. The plates were then incubated further at 27^{o}C for 48 hours. The control wells contained culture alone. 10μl of 2, 5-diphenyltetrazolium bromide (MTT) reagent was added into all the wells and incubated for 4 hours. The medium together with MTT were aspirated off, followed by addition of 100μl of dimethyl sulfoxide (DMSO) per well and shaken for 5 minutes. Absorbance was measured for each well at 562 nm using a micro-titer reader. The absorbance readings were used to generate the 50% inhibitory concentration (IC_{50}) values for extracts A, B, and C. Percentage promastigotes' viability (%) was determined using the formula described by Mosmann (1983), in which, viable promastigotes (%) = $(AT - AB) / (AC) \times 100$, where *AT* was the absorbance of treated samples and *AB* was the absorbance of the blank wells and *AC* was the absorbance of the control wells.

Determination of IC_{50} of extracts using Vero cells

The assay was carried out as described by Wabwoba *et al*. (2010). The vero cells were harvested by trypsinization, and pooled in 50 ml centrifuge tubes from where a 100μl of the cell suspension were put

into 2 wells of rows A-H in a 96-well flat bottomed microtiter plate at a concentration of 1×10^6 cells per ml of the culture medium per well and incubated at 37°C in 5% Co_2 in order to attach. The MEM was gently aspirated off and 150 μl of the highest concentration (1000μg/ml) of the test extracts (extracts A, B, C,) was added and serially diluted by a factor of two up to a concentration of 15.63μg/ml. The microtitre plates containing the vero cells and test extracts were further incubated at 37°C for 48 hours in a humidified 5% CO_2 atmosphere. The controls wells comprised of vero cells and medium only while the blank wells had medium alone. Ten microliters of MTT reagent was added into each plate well and incubated further for 2 to 4 hours until a purple precipitate (formazan) was visible under the microscope. The media together with MTT reagent were gently aspirated off, after which 100 μl of dimethyl sulfoxide (DMSO) was added, and vigorously shaken for 5 minutes in order to dissolve formazan. The absorbance (optical density) was measured for each well plate using a micro-titer plate reader at wavelength of 570 nm. The IC_{50} values of the extracts were determined automatically using the Chemosen Software Program.

Infection and treatment of BALB/c mice

The infection of groups of mice was carried out as described by Wabwoba *et al*., 2010. Each group comprised of at least 5 mice. The thickness of the hind legs footpads was measured using a vernier caliper prior to infection. The left hind footpads of the mice were subcutaneously inoculated with 1×10^6 stationary phase infective metacyclic promastigotes of *L. major* in 40μl sterile PBS. Groups of infected mice were treated for four weeks with the aqueous crude extracts A, B, and C one month post inoculation. Treatment was done orally using a cannula and intra-peritoneally using a fine 1ml 30 gauge insulin needles (BD Micro-Fine Plus®, USA) at a dose of 20 mg/kg of the extracts daily. The positive control groups of mice were treated intra-peritoneally with pentostam and liposomal amphotericin B at a dose of 20 mg/kg per day. The foot pads lesions progression was monitored weekly using a vernier caliper to measure the thickness of the infected left hind foot pad and to compare it with non infected right hind foot pad as described by Nolan & Farrel (1987).

Parasite burden in spleens from treated BALB/c mice

After a four week treatment period, the mice were sacrificed using 100μl pentobarbitone sodium (Sagatal®). At necropsy, the spleens were weighed and spleen impression smears were made as described by Chulay & Bryceson (1983). The spleen impression smears were fixed in methanol and stained with Giemsa. The smears were examined under a microscope to enumerate the number of amastigotes per 1000 nucleated spleen cells. The relative and total numbers of parasites in the spleen were estimated by calculating the spleen index (%), Leishman-Donovani Units (LDUs) and Total Leishman-Donovani Units (Total LDUs) as described by Bradley & Kirkley (1977). Spleen index (%) was determined using the formula: Spleen weight (g) divided by mouse body weight × 100%. LDU was the number of amastigote per 1000 nucleated splenocytes while the total LDU was calculated by formula: LDU × mouse spleen weight (g) × (2×10^5).

Data analysis

Data was analyzed using SPSS for windows at 5% level of significance. One way ANOVA (F test) was used to compare the lesion sizes in groups of mice under different treatments. Other variables compared using F test were LDU and total LDU for mice under different treatments. Cases in which homogeneity test of variance (Levene's test) were significant, robust tests of equality of means that included Brown-forsythe and Welch tests were carried out as alternative versions of the F-statistics. Multiple comparisons of the individual treatments were done using both Tukey HSD and Games-Howell *post hoc* tests.

Results

Plant extracts yields

A. sativum and *C. citrinus* aqueous yields were highest at 18.59 % and 17.58 % respectively as shown in Table 1.

Table 1: The percentage yields of the plant extracts obtained from the study plants.

Plant species	Part used	Code	Initial wt (g)	Yield (g)	Yield (%)
A. sativum	Bulbs	A	50	9.293	18.59
C. citrinus	Flowers	B	50	8.790	17.58
M. stenopetala	Leaves	C	50	3.200	6.40

IC_{50} of aqueous extracts when measured against *L. major promastigotes* and Vero cells

The aqueous extracts of *M. stenopetala*, *C. citrinus*, and *A. sativum* had IC_{50} values that ranged from 297μg/ml to 575μg/ml as compared to Pentostam and liposomal amphotericin B which had IC_{50} values of 0.26μg/ml and 0.82μg/ml respectively (Table 2). Low IC_{50} corresponded to low viability (survival) of the *L. major* promastigotes *in vitro* as shown in Table 2. On a scale of 10, the control drugs were more effective as indicated by their high $-\log_{10}IC_{50}$ value (pIC_{50} scale). There was significant difference between the $-\log IC_{50}$ values for the aqueous extracts and those of control drugs ($P < 0.05$).

Table 2: Showing IC_{50} of test extracts, MIC and viability (%) of *L. major* promastigotes after *in vitro* treatment with the extracts or the controls.

Test extracts & controls	Code	MIC (mg/ml)	IC_{50} (μg/ml)	$-\log_{10}IC_{50}$ (pIC_{50} scale)	Viability (%)
Aqueous[a]					
M. stenopetala	A	3	299.79	-2.48	52.55
C. citrinus	B	5	297.75	-2.47	75.74
A. sativum	C	5	575.75	-2.76	60.57
Positive controls[b]					
Pentostam	Pent	0.0125	0.26	0.59	18.41
Lip amphotericin B	Am B	0.0063	0.82	0.09	12.22
Negative control					
Schneider's Medium	SIM	-	-	-	81.65

[a] = the concentration of the extracts ranged between 5mg/ml to 0.5mg/ml; [b] = the initial concentration was 100 μg/ml (0.1mg/ml) serially diluted by a factor of 2.

Similarly, aqueous extracts of *C. citrinus* (B) were slightly more toxic as compared to those of aqueous extracts of *A. sativum* (C) and *M. stenopetala* (A) as shown in Table 3. The leishmaniases drugs pentostam and liposomal amphotericin B were more toxic when compared to the extracts.

Table 3: The toxicity of the aqueous extracts measures as IC_{50} (μg/ml) against vero cells

Test extracts	Code	IC_{50} (μg/ml)
Aqueous:		
M. stenopetala	A	1306.68
C. citrinus	B	467.11
A. sativum	C	2105.93
Control drugs:		
Pentostam	Pento	108.58

Liposomal Amphotericin B	Amph B	60.95

Note: The initial concentration of the test extracts was 1000μg/ml while that of control drugs was 100 μg/ml

Effects of aqueous extracts on *L. major* caused footpad lesions

Orally administered *M. stenopetala* extracts (A) efficacy was low however it caused the foot pad lesion sizes stabilize from week 4 of treatment while oral aqueous *C. citrinus* extracts (B) caused a steady decrease of the foot pad lesions from week 3 of treatment, a trend that was closely comparable to efficacy of drug Pentostam (Figure 1). Oral *A. sativum* reduced the footpad lesion sizes significantly when compared to the mice treated with PBS ($P < 0.05$).

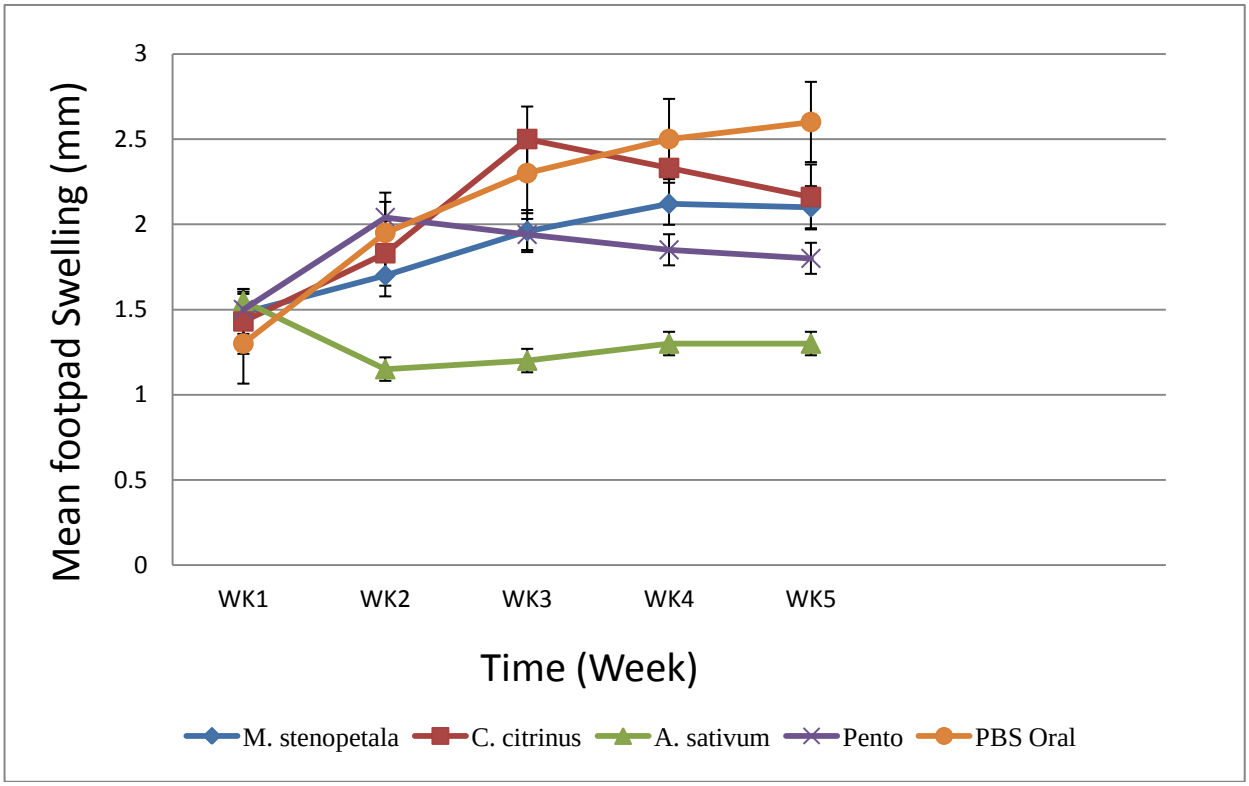

Figure 1: The foot pad swelling after oral treatment of *L. major* infected BALB/c mice with test aqueous extracts. Pento = Pentostam; PBS oral = phosphate buffered saline administered orally.

As shown in Figure 2, the aqueous *A. sativum* extracts (C) when administered intra-peritoneally (ip) reduced the footpad swelling for the first three weeks of treatment. Both the aqueous extracts of *C. citrinus* (B) and *M. stenopetala* (A) limited the footpad lesion progression from the third week of treatment onwards. There was significant difference ($P < 0.05$) between the mean lesion size of mice treated with PBS (ip) and those treated with aqueous extracts B (ip) and aqueous extracts C (ip).

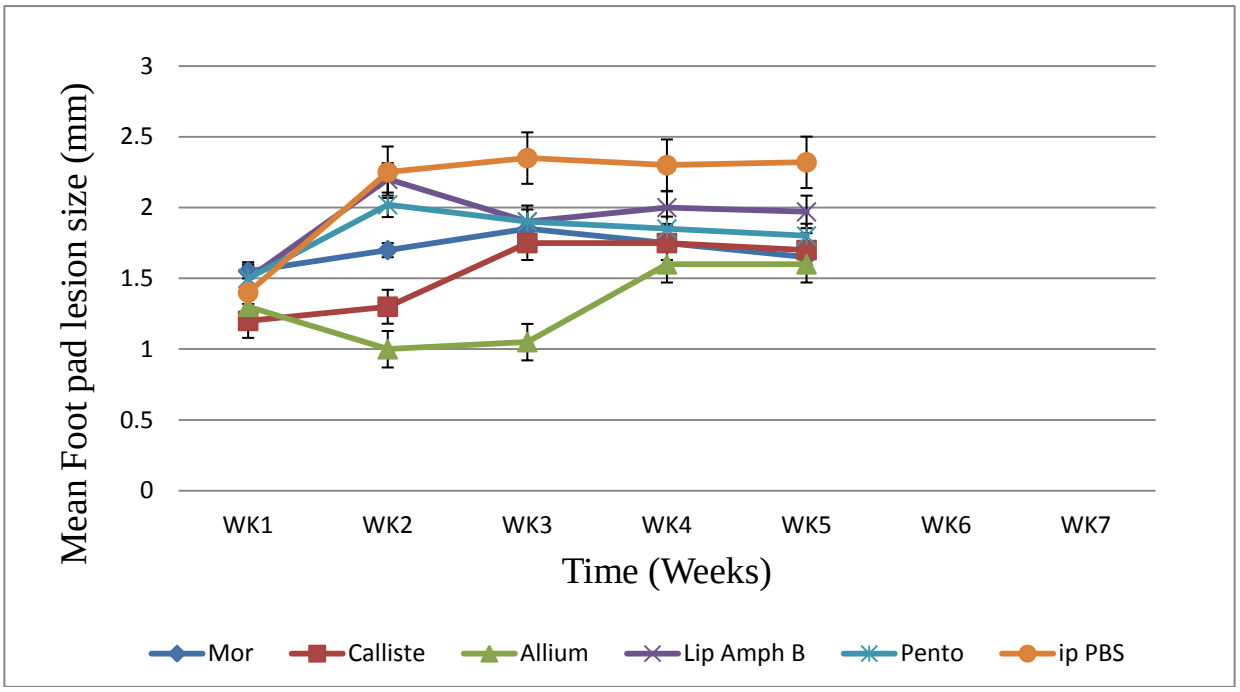

Figure 2: The foot pad swelling after intra-peritoneal treatment of *L. major* infected BALB/c mice with aqueous extracts and control drugs. Mor = *M. stenopetala*; Calliste = *C. citrinus*; Allium = *A. sativum*; Amph-B= liposomal amphotericin B; Pento = Pentostam; ip PBS = intra peritoneally administered phosphate buffer saline.

Estimation of *L. major* amastigotes in the splenocytes of treated BALB/c mice

Table 4 shows that, the efficacy of oral *C. citrinus* extracts (B) in reducing parasite load in the spleens of *L. major* infected BALB/c mice was the highest at 82.99%, followed by oral *M. stenopetala* (A) at 66.96% and oral *A. sativum* (C) at 60.37%. In comparison, pentostam and liposomal amphotericin B reduced the amastigotes by 66.40% and 60.62% respectively. The infected mice treated with negative controls (PBS) had high parasite burdens in their spleens as indicated by the low % parasite reduction (Table 4). There was a significant difference (F (df: 3, 8) = 6.313, P = 0.017) between the mean total LDU for aqueous oral *C. citrinus* extracts and the mean total LDU for control oral PBS. The orally administered extracts were associated with a low total LDUs and high % parasite reduction (Figure 3).

Table 4: The average spleen index ± SE, LDU ± SE and total LDU ± SE for groups of *L. major* infected BALB/c mice that were treatment with single aqueous and test extracts, pentostam, liposomal amphotericin B and PBS controls.

Test extracts & controls	Route	Ave spleen index (%)	Ave LDU	Ave total LDU (× 1000)	% parasite reduction [a]
Aqueous:					
A (*M. stenopetala*)	oral	0.51 ± 0.14	0.25 ± 0.04	4.06 ± 0.60	66.96
	ip[b]	0.54 ± 0.01	0.57 ± 0.05	10.28 ± 2.02	16.35
B (*C. citrinus*)	oral	0.71 ± 0.21	0.10 ± 0.02	2.09 ± 0.14	82.99
	ip	0.53 ± 0.03	0.19 ± 0.13	3.05 ± 2.02	75.18
C (*A. sativum*)	oral	0.42 ± 0.01	0.27 ± 0.01	4.87 ± 0.21	60.37
	ip	0.47 ± 0.05	0.33 ± 0.24	6.71 ± 4.80	45.40
Controls:					

Pentostam	ip	0.73 ± 0.19	0.18 ± 0.08	4.13 ± 1.10	66.40
Lip amph B	ip	0.61 ± 0.02	0.24 ± 0.02	4.84 ± 0.38	60.62
PBS	ip	0.54 ± 0.04	0.38 ± 0.21	8.74 ± 5.30	28.89
PBS	oral	0.56 ± 0.06	0.61 ± 0.22	12.29 ± 4.49	00.00

[a] means that the % was calculated in reference to total LDU for PBS oral which was taken to represent 100% parasite burden; ip[b] = means intraperitoneal.

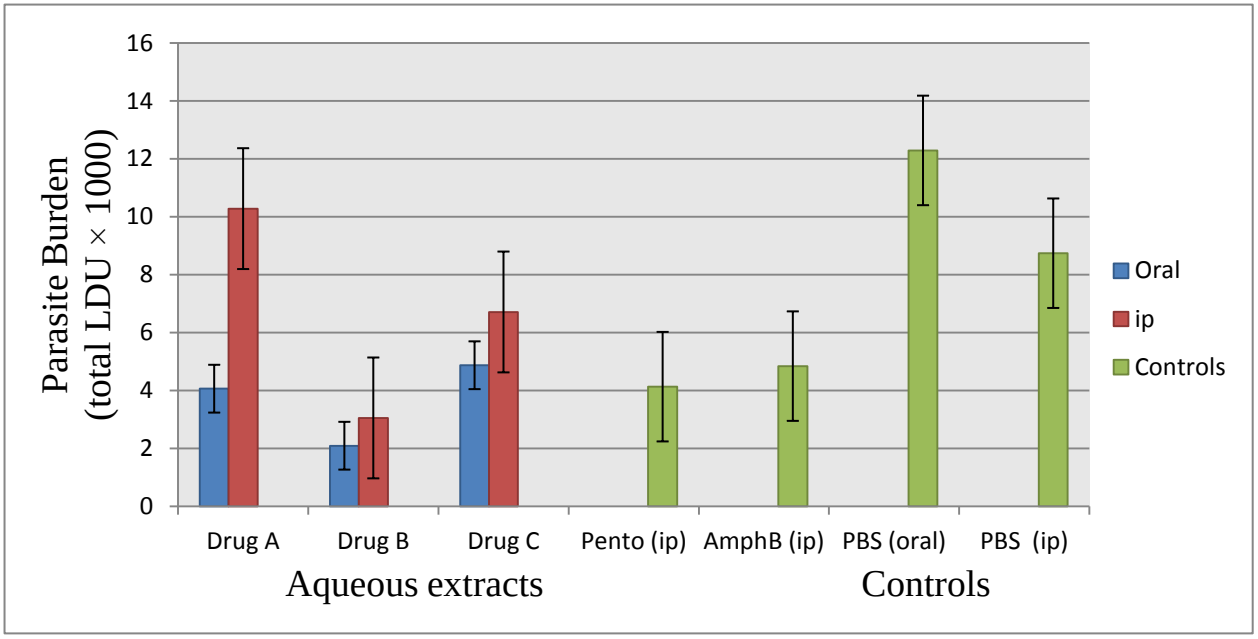

Figure 3: Parasite burden in spleens of *L. major* infected BALB/c mice treated with test extracts, pentostam, liposomal amphotericin B, and PBS. The extracts were A = aqueous *M. stenopetala*; B = aqueous *C. citrinus*; C = aqueous *A. sativum* extract; while the controls were Pento = pentostam; AmphB = liposomal amphotericin B; PBS= phosphate buffered saline.

Discussion

The present study shows that aqueous extracts of moringa, bottlbrush and garlic plants reduced the viability of *L. major* promastigotes *in vitro* by about 25 to 50% as compared to more than 80% by pentostam an liposomal amphotericin B. Although the standard drugs were much more effective against *Leishmania* promastigotes, they have been reported to be highly toxic to patients and yet they require a protracted administration (Martinez & Marr, 1992; Santos *et al*., 2008). Cytotoxic assays using vero cells in the current study showed that the test aqueous extracts were similarly less toxic compared to the pentostam and liposomal amphotericin B. The continued use of the contemporary leishmaniases drugs despite their high toxicity is mainly due to lack of an alternative. The use of herbal materials can be a cheaper, safer and readily available alternative. Previous studies have shown that aqueous *C. citrinus* extracts become toxic in rats in high dosage (Firoz *et al*., 2011). Extracts of *C. citrinus* are rich in essential oils that include 1,8-cineole and alpha pinene (Oyedeji *et al*., 2009) and according to Koul *et al*. (2008), the terpenoid constituents of the essential oils can be moderately toxic to mammals. 1,8-cineole was shown to have a toxicity level of LD_{50} 2480 mg/kg when administered orally in experimental rats (Koul *et al*., 2008). The moderate *in vitro* cytotoxicity of *C. citrinus* extracts to *L. major* promastigotes and vero cells observed in the present study was probably due to its essentials oils.

Previous reports have shown that aqueous *A. sativum* extracts possess cytotoxic effects on *L major* promastigotes at an IC_{50} level of 37 mg/ml (Gharavi *et al*., 2011). In the current study however, the IC_{50} of aqueous *A. sativum* extracts were 0.58 mg/ml respectively in the same duration of 48 hours. The difference could have been attributed to the source and the primary processing of the garlic used for the experiment. Gharavi and colleagues used fresh garlic while in the present study, dry garlic was used. Garlic has sulphur containing compounds, all of which have medical benefits. Among these compounds are allicin (diallyl thiosulfinate) which is the most abundant and it gives garlic the characteristic odor. These compounds seem to be relatively less toxic from the current study. The aqueous extracts of *M. stenopetala* were relatively less toxic to *L. major* promastigotes and vero cells compared to pentostam and liposomal amphotericin B. According to Mekonnen *et al*. (2005), ethanol extract of the leaves and seeds from *Moringa stenopetala* contain toxic substances. These substances may have contributed to the slight toxicity observed in the present study. The low toxicity of *M. stenopetala* may explain why the plant is used as a nutrient rich vegetable in southern Ethiopia.

Mice treated with oral aqueous *M. stenopetala* extracts (A) either had their footpad lesion sizes stabilize from week four of treatment. When administered intra peritoneally, the aqueous moringa extracts stabilized the lesion sizes also from week three of treatment. Several properties of *M. stenopetala* extracts which could contribute to speedy wound healing include having a lot of nutrients especially proteins which are required to make new tissues. Moringa extracts are also known to be immune builders, antiulcers and antibacterial which are all necessary for speedy tissue healing. *M. stenopetala* are rich in bioactive antibiotic glucosinolates compounds (Fahay, 2005; Bellostas *et al*., 2010). This could explain the plant's *in vivo* inhibitory activity against *L. major* amastigotes in BALB/c mice spleens, observed in the current study. In our previous study, aqueous extracts of *M. stenopetala* were shown to be active against *L. major* amastigotes *in vitro* (Kinuthia *et al*., 2013).

Orally administered aqueous *C. citrinus* extracts (B) reduced lesion sizes significantly when compared to PBS (oral). Similarly intra peritoneally administered extracts B limited the footpad lesion sizes from week three of treatment onwards. *Callistemon* extracts have previously been reported to be strong broad spectrum antibacterial agents and this would have contributed in inhibiting the bacterial effects in the lesion wounds hence accelerating their healing. The *C. citrinus* extracts may have destroyed the amastigotes in the lesions directly. *In vivo* efficacy of *Callistemo*n extracts in reducing *L. major* amastigotes was high as indicated by the low total LDUs hence suggesting that the essential oils present in the *C. citrinus* extracts (Oyedeji *et al*., 2009) possess antiprotozoa activity.

Aqueous *A. sativum* (C) reduced the foot pad lesion sizes significantly implying that the extracts activated wound healing. Garlic extracts have previously been reported to cause re-epithelialization of exposed wounds and they also increase the number of loosely packed collagen and maturation of collagen bundles (Ejaz *et al*., 2009). The present results show that the aqueous garlic extracts reduced the amastigotes burden in the treated BALB/c mice spleens. This suggests that garlic also possess antiprotozoa activity *in vivo*. The medicinal property of garlic has been attributed to its organosulfur compounds which include alliin, allicin, diallyl sulfide, diallyl disulfide, ajoene among others (Islam *et al*., 2011). According to McClure *et al*. (1996), allicin (diallyl thiosulfinate) inhibits leishmanial cell growth significantly.

Conclusion

Crude aqueous extract of *M. stenopetala* ((Baker F) Cufodontis, *A. sativum* L (garlic), and C. citrinus (Curtis) Skeels are relatively less toxic when tested against vero cells *in vitro* however they are active against *L. major* promastigotes and amastigotes. In particular aqueous extracts of dry garlic reduced *L. major* caused foot pad lesions significantly when compared to the efficacy of phosphate buffered saline

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References

Abdelhady, M. I. & Aly, H. A. H. (2012). Antioxidant and antimicrobial activities of *Callistemon comboynensis* essential oils. *Free Radicals and Antioxidants*; **2** (1): 37 – 41.

Ali, N., Shah, S. W. A. & Ahmad, B. (2010). Calcium channel blocking of fruits of *Callistemon citrinus*. *Journal of the chemical Society of Pakistan*; **33** (2): 245 – 248.

Alvar J.,Valez ID., Bern C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., WHO leishmaniasis control team. (2012). Leishmaniasis worldwide and global estimates of its incidences. *PLoS ONE*; **7** (5): e 35671. Doi: 10.1371/journal.pone. 0035671.

Anez, N., Carrasco, H. & Parada H. (1999). Acute Chaga's disease in western Venezuela: A clinical seroparesitologic and epidemiological study. *American Journal of tropical medicine & hygiene*; **60**: 215 – 222.

Augusti, K. T. (1996). Therapeutic Values of onion (*Allium cepa* L.) and garlic (*Allium sativum* L). *Indian Journal of Experimental Biology;* **34**: 634 - 640.

Bellostas, N., Sorensen, J. C., Nikiema, A., Sorensen, H., Pasternak, D. & Kumar, S. (2010). Glucosinolates in leaves of *Moringa* species grown and disseminated in Niger. *African Journal of Agricultural Research*; **5** (11): 1338 – 1340.

Bradley, D.J. & Kirkley, J. (1977). Regulation of *Leishmania* population within the host. The variable course of *Leishmania donovani* infections in mice. *Clinical Experimental Immunology*; **30**: 119 – 129.

Chulay, J. D. & Bryceson, A. D. M. (1983). Quantification of amastigotes in smears of splenic aspirates from patients with visceral leishmaniasis. *American Journal of Tropical Medicine and Hygiene*; **32**: 3475 – 3479.

Clem, A. (2010). A current perspective on leishmanasis. Journal of global infectious diseases; 2(2): 124 – 126.

Delahaye, C., Rainford, L., Nicholson, A., Mitchell, S., Lindo, J. & Ahmad, M. (2009). Antibacterial and antifungal analysis of crude extracts from leaves of *Callistemon viminalis*. *Journal of Medical and Biological Sciences;* **3**(1): ISSN 1934 – 7189.

den Boer, M., Argaw, D., Jannin, J. & Alvar, J. (2011). Leishmaniasis impact and treatment access. *Clinical Microbiology and Infection*; 17(10): 1471 – 1477.

Dongmo, B. N., Dongmo, P. M. J., Ngoune, L. T., Kwazou, N. L., Zollo, P. H. A. & Menut, C. (2010). Antifungal activities of essential oils of some Camerounian Myrtaceae on *Aspergillus flavus* Link ex. Fries. *Asian Journal of Experimental Biological Sciences*; **1** (4): 907 – 914.

Ejaz, S., Chekarova, I., Cho, J. W., Lee, S. Y., Ashraf, S. & Lim, C. W. (2009). Effect of aged garlic extract on wound healing: a new frontier in wound management. *Drug and chemical toxicology*; **32**(3); 191 – 203. Doi: 10. 1080/01480540902862236.

Fahey, J. W. (2005). *Moringa oleifera*: A review of the medicinal evidence for its nutritional, therapeutic and prophylactic properties: Part I. *Trees for life Journal*; **1**: 5.

Firoz, N., Bharatesh, K., Nilesh, P., Vijay, G., Tabassum, S. & Nilofar, N. (2011). Cardioprotective activity of ethanolic extract of *Callistemon lanceolatus* leaves on doxorubicin cardiomyopathy in rats. *Bangladesh Journal of Pharmacology*; **6** (1): 38 – 45.

Gathirwa, J. W., Rukunga, G. M., Njagi, E.N.M., Omar, S. A., Guantai, A. N., Tolo, F. M., Kimani, C. W., Muthaura, C. N., Kirira, P. G., Ndunda, T. N., Amalemba, G., Mungai, G. M. & Ndiege, I. O. (2008). The *in vitro* anti-plasmodial and *in vivo* anti-malarial efficacy of combinations of some medicinal plants used traditionally for treatment of malaria by the Meru Community in Kenya. *Journal of Ethno-pharmacology*; **115**: 223 – 231.

Gharavi, M. J., Nobakhr, M., Khademvatan, S. H., Bandani, E., Bakhshayesh, M. 7 Roozbehani, M. (2011). The effect of garlic extract on expression of IFN-γ and INos genes in macrophages infected with *Leishmania major*. *Iranian Journal of Parasitology*; **6** (3): 74 – 81.

Gicheru, M. M., Olobo, J. O., Anjili, C. O., Orago, A. S., Modabber, F. and Scott, P. (2001). Vervet monkeys vaccinated with killed *Leishmania major* parasites and interleukin -12 develop a type I immune response but are not protected against challenge infection. *Infection and Immunity*; **69**(**1**): 245-251.

Hendricks, L. D. & Wright, N. (1979). Diagnosis of Cutaneous Leishmaniasis by *In Vitro* Cultivation of Saline Aspirates In Schneider's *Drosophila* Medium. *American Journal of Tropical Medicine & Hygiene*; **28**: 962 - 964.

Islam, M. S., Kusumoto, Y. & Abdulla Al-Mamun, M. (2011). Cytotoxicity and cancer (HeLa) killing effeicacy of aqueous garlic (*Allium sativum*) extract. *Journal of Scientific Research*; **3**(2): 375-382.

Joy, P. P., Thomas, J., Mathew, S. & Skaria, B. P. (2001). Medicinal Plants. *Tropical Horticulture Volume 2*. (eds. Bose, T. K., Kabir, J., Das, P. & Joy, P. P.). Naya Prokash, Calcutta, 449 – 632.

Kigondu, E. V. M., Rukunga, G. M., Keriko, J. M., Tonui, W. K., Gachirwa, J. W., Kirira, P. G., Irungu, B., Ingonga, J. M. & Ndiege, I. O. (2009). Anti-Parasitic Activity And Cytotoxicity Of Selected Medicinal Plants From Kenya. *Journal of Ethnopharmacology*; **123**(3): 504 – 509.

Kimber, C. D., Evans, D. A., Robinson, B. L. & Peters, W. (1981). Control of yeast contamination with 5-fluorocytosine in the *in vitro* cultivation of *Leishmania spp*. *American Journal of Tropical Medicine & Parasitology*; **75**: 453 – 454.

Kinuthia, G.K., Anjili, C.O., Gikonyo, N.K., Kigondu, E.M., Ingonga, J.M. & Kabiru, E.W. (2013). *In vitro* and *in vivo* activities of blends of crude aqueous extracts from *Allium sativum* L, *Callistemon citrinus* (Curtis) Skeels and *Moringa stenopetala* (Baker F) Cufodontis against *Leishmania major*. *International Journal of Medicinal and Aromatic Plants*; **3**(2): 234 – 246.

Koul, O., Walia, S. & Dhaliwal, G. S. (2008). Essential oils as green pesticides: Potential and constraints. *Biopesticides International*; **4**(1): 63 – 84.

Martinez, S. & Marr, J. J. (1992). Allopurinol In The Treatment Of American Cutaneous Leishmaniasis. *The New England Journal of Medicine*; **326**: 741 – 744.

McClure, C. D., Linda, L. N. & Zatyrka, S. A. (1996). Antileishmanial properties of *Allium sativum* extracts and derivatives. *ISHS Acta Horticulture 426* : International symposium on medicinal and aromatic plants.

Mekonnen, Y., Yardley, V., Rock, P & Croft, S. (1999). *In vitro* anti-trypanosomal activity of *Moringa stenopetala* leaves and roots. *Phytotherapy Resources;* **13**: 538 – 539.

Mekonnen, Y. & Drager, B. (2003). Glucosinolates in *Moringa stenopetala*. *Planta Medica*; **69**: 380 – 382.

Mekonnen, N., Houghton, P. & Timbrell, S. (2005). The toxicity of extracts of plant parts of *Moringa stenopetala* in HEPG2 cells *in vitro*. *Phytotherapy Resources*; **19**(10): 870 – 875.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*; **16**: 55 – 63.

Nolan, T. J. & Farrel, J. P. (1987). Experimental Infections of the Multimammate rat (*Mastomys natalensis*) with *L. Donovani* and *L. Major*. *American Journal of Tropical Medicine & Hygiene;* **36**(2): 264 – 269.

Oyedeji, O. O., Lawal, O. A. Shode, F. O. & Oyedeji, A. O. (2009). Chemical composition and Antibacterial activity of the essential oils of *Callistemon citrinus* and *Callistemon viminalis* from South Africa. *Molecules*; **14**(6): 1990 – 1998.

Santos, D. O., Coutinho, C. E. R., Bottino, C. G., Viera, R. T., Nascimento, S. B., Bourguignon, S. C., Castro, H. C., Madeira, M. F., Bernardino, A., Corte-Real, S., Pinho, R.T. & Rodrigues, C. R. (2008). Leishmaniasis treatment – a challenge that remains: a review. *Parasitology Research*; **103**: 1 – 10.

Tahany, M. A. A**.,** Hegazy, A. K., Sayed, A. M., Kabiel, H. F., El-Alfy, T & El- Komy, S. M. (2010). Study on combined antimicrobial activity of some biologically active constituents from wild *Moringa peregrine* Forssk. *Journal of Yeast and Fungal Research*; **1**(1): 15 – 24.

Wabwoba, B., Anjili, C. O., Ngeiywa, M. M., Ngure, P. K., Kigondu, E. M., Ingonga, J. & Makwali, J. (2010). Experimental Chemotherapy with *Allium Sativum* (Liliaceae) Methanolic Extract in Rodents infected with *Leishmania major* and *Leishmania donovani*. *Journal of Vector Borne Diseases*; **47**: 160 – 167.

Yousefi, R., Ghaffarifar, F. & Asl, A. D. (2009). The effect of *Alkanna tincturia* and *Peganum harmala* extracts on *Leishmania major* (MRHO/IR/75/ER) *in vitro*. *Iranian Journal of Parasitology*; **4**(1): 40 – 47.