Int. J. Med. Arom. Plants, ISSN 2249 – 4340
Vol. 3, No. 2, pp. 234-246, June 2013

RESEARCH ARTICLE

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*

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Article History: Received 17th April 2013, Revised 17th June 2013, Accepted 18th June 2013.

Abstract: *Leishmania major* caused cutaneous leishmaniasis leads to painful skin sores in humans and usual drugs are expensive, toxic, and require prolonged use. The *in vitro* and *in vivo* efficacy of aqueous crude extracts from *Callistemon citrinus* flowers (B), *Allium sativum* bulbs (C) and *Moringa stenopetala* leaves (A) against *L. major* was studied. Controls were pentostam, liposomal amphotericin B, and phosphate buffered saline (PBS). Dried and ground plant materials were soaked in distilled water at 70^{o}C for 1.5 hours, filtered and freeze dried to obtain aqueous extracts. *L. major* infected BALB/c mice were treated orally or intra peritoneally (ip) with blends of the extracts. Minimum inhibitory concentrations (MICs) of single extracts ranged from 3 to 5mg/ml while IC_{50} from 297 to 575μg/ml compared to MICs of 12.50 and 6.25μg/ml and IC_{50} of 0.26 and 0.82μg/ml for pentostam and liposomal amphotericin B respectively. Blends of *M. stenopetala* and *C. citrinus* (AB), *M. stenopetala* and *A. sativum* (AC), and *C. citrinus* and *A. sativum* (BC) at concentrations based on MICs of individual extracts were active at ratios 1:1, 1:9 and 1:1 with promastigotes' viabilities of 33.82%, 17.41% and 60.74 % respectively. IC_{50} for blends AB, AC, and BC ranged from 174μg/ml to 1314μg/ml against promastigotes. The individual extracts comprising blends AB, AC and BC interacted additively and synergistically in several combination ratios. Blend AC (1:1) at 125µg/ml had *in vitro* infection rate (IR) of 71% and multiplication index (MI) of 48.20% for *L. major* amastigotes compared to IR of 67% and MI of 47.51% for pentostam at 12.50μg/ml. Oral blend BC (1:1) reduced the mice footpad lesion size significantly ($P < 0.05$). Both oral blends BC and AC reduced mice spleen amastigotes by 48.33% and 60.94% with total LDUs of 6.35 ± 0.66 and 4.80 ± 0.95 respectively. Oral blend AB (1:1) lowered spleen amastigotes by 6.5% with total LDU of 11.49 ± 6.84. In conclusion, aqueous blends of *C. citrinus*, *A. sativum* and *M. stenopetala* extracts that interacted additively or synergistically were less toxic but active against *L. major*.

Keywords: *Allium sativum*; *Callistemon citrinus*; *Moringa stenopetala*; *Leishmania major*; antileishmanial.

Introduction

Leishmaniasis is an infection caused by a protozoan parasite belonging to the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) that is spread to people through the bite of the female phlebotomine sandfly. Cutaneous leishmaniasis (CL) is the most common form which is endemic in more than 70 countries worldwide (Reithinger *et al*., 2007) in four continents and with about 1.5 million people being infected every year in the world (Patil *et al*., 2012). CL causes skin ulcers that develop at the

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site of the sand fly bite, pain and often it is associated with marked disfigurement if multiple lesions accumulate (Polonio and Efferth, 2008). In Africa, Asia Minor and Europe, the causative agent for cutaneous leishmaniasis is *L. major*, *L. aethiopica*, *L. tropica*, *L. infantum* and *L. killicki* (Reithinger *et al*., 2007; Aoun *et al*., 2008). Ninety percent of CL cases have been reported in Afghanistan, Syria, Saudi Arabia, Brazil and Peru (Tonui, 2006). In Kenya, CL is also common in Baringo, Kitui, Kiambu, Laikipia, Samburu, Nakuru, Nyandarua, and Mt Elgon areas (Tonui, 2006). For six decades, pentavalent antimonials in form of sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime), administered intravenously or intramuscularly, have been used as first line treatment of all types of leishmaniasis (Polonio and Efferth, 2008). Other leishmaniasis drugs include pentamidine, paromomycin sulfate, amphotericin B, liposomal amphotericin B, and oral miltefosine. Pentavalent antimonials are highly toxic, expensive and require a prolonged administration and hospitalization of the patient. Cases of high level antimonial and pentamidine resistant leishmaniases have been reported in India (Chakravarty and Sundar, 2010). Multidrug combination therapy has been used to prevent the emergence of drug resistance, lower the dosage required and hence the toxicity levels, reduce costs incurred, and increase the spectrum of the drugs activity (Chakravarty and Sundar, 2010). Combination of drugs may be associated with increased activity because of the synergistic and additive interactions. In Sudan, paromomycin in combination with sodium stibogluconate (Pentostam) was reported to be more efficacious than the sodium stibogluconate alone (Melaku *et al*., 2007).

The herbal products are increasingly becoming important because they symbolize safety in contrast to the synthetic drugs (Joy *et al*., 2001). Natural compounds that include alkaloids, terpenes, and phenolic derivatives obtained from a variety of plants, have been reported to show anti-leishmanial activities (Patil *et al*., 2012). Use of herbal products as combinations (blends) is a common practice in Kenya and it has existed in many cultural systems for centuries (Gathirwa *et al*., 2008). Previously, combination therapy has been reported to be effective against leishmaniasis.

Callistemon citrinus (Curtis) Skeels popularly known as the bottle brush is an ornamental tree in Kenya, and it possesses antimicrobial, nematocidal, larvicidal, and pupicidal activities (Ali *et al*., 2010) and anti-fungal property (Dongmo *et al*., 2010). *Allium sativum* L (Garlic) has been used as food, spice and medicine for thousands of years (Islam *et al*., 2011) and its medicinal properties range from antimicrobial, antiviral, antifungal to antiparasitic activities (Goncagul *et al*., 2010). *Moringa stenopetala* (Baker F) Cufodontis commonly referred to as the African Moringa and whose leaves and fruits are eaten in Ethiopia as vegetables is rich in proteins, calcium, phosphorous, iron and vitamins A and C. African Moringa possesses antimicrobial activity (Eilert *et al*., 1980), antimalaria and antitrypanomastigote properties and it is used to treat stomach problems (Mekonnen *et al*., 1999). It is against this background that the present study was designed to investigate the *in vitro* and *in vivo* efficacy of blends of aqueous crude extracts from *C. citrinus*, *A. sativum* and *M. stenopetala* against *L. major*.

Materials and methods

Plant materials

The *C. citrinus* flowers were harvested from randomly selected homesteads in Nakuru County, Kenya. Bulbs of *A. sativum* were purchased from Nakumat super market in Nairobi, Kenya. *M. stenopetala* young leaves were collected from one of the islands in Lake Baringo, Kenya. The plants were positively identified at University of Nairobi herbarium, in the department of Botany at Chiromo campus. The *C. citrinus* flowers, thin slices of *A. sativum* cloves and young leaves of *M. stenopetala* were taken to Kenya Medical Research Institute (KEMRI) *Leishmania* laboratory, Nairobi, where they were dried at room temperature until they became brittle and attained a constant weight.

Plant extracts preparation

The dried plant materials were labeled appropriately and then transferred to the Center of Traditional Medicine and Drug Research (CTMDR) at KEMRI, where they were separately ground using an electrical mill (Christy and Norris Ltd, England) into powder, followed by extraction using water. 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 then filtered using Whatman No 1 filter papers and the filtrate was freeze dried. The aqueous extracts of *M. stenopetala*, *C. citrinus*, and *A. sativum* were coded as extracts A, B and C respectively.

Leishmania parasites

The *Leishmania major* strain (IDUB/KE/94=NLB-144) was acquired from Institute of Primate Research (IPR), Kenya where it had been cryo preserved 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 (FBS), 100 U/ml penicillin and 500μg/ml streptomycin (Hendricks and Wright, 1979), and 250μg/ml 5-fluorocytosine arabinoside (Kimber *et al*., 1981). The stationary-phase metacyclic promastigotes were then harvested by centrifugation at 1500 rpm for 15 minutes at 4^{o}C. The metacyclic promastigotes were then used for *in vitro* and *in vivo* assays.

Experimental animals

Inbred BALB/c mice were obtained from International Livestock Research Institute (ILRI), Kenya. They were housed at the KEMRI animal house at 23^{o}C to 25^{o}C and were fed on standard commercial diet that was in form of mice pencils and given tap water *ad libitum*. The mice were handled in accordance with the regulations that have been set by Animal Care and Use Committee (ACUC) at KEMRI. Eight week old male mice were used for both *in vitro* and *in vivo* assays.

Evaluation of minimum inhibitory concentration (MIC)

The MICs was determined as described by Wabwoba *et al*., (2010). The *L. major* promastigotes at a concentration of 1×10^{6} promastigotes per ml were grown in Schneider's insect medium in 24 well micro titer plate containing the test aqueous extracts (A or B or C) in concentrations that ranged between 5mg/ml and 0.5mg/ml. Survival of the promastigotes upon exposure to seven different fixed ratios ranging from 9:1 to 1:9 for blends AB (*M. stenopetala* : *C. citrinus*), AC (*M. stenopetala* : *A. sativum*), and BC (*C. citrinus* : *A. sativum*) was also evaluated. The lowest concentration of the test plant extracts that prevented or inhibited promastigotes growth was taken to be MIC and the blends ratio that supported the least survival or total inhibition of promastigotes growth was noted.

Promastigote proliferation measurement by colorimetric MTT assay

MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) colorimetric assay was used to measure the reduction of MTT dye (tetrazolium) into formazan by mitochondrial enzymes in viable promastigotes. The procedure was carried out as described by Wabwoba *et al*. (2010). Promastigotes at a concentration of $1x10^{6}$ promastigotes per ml were grown for 48 hours in 24 well micro titer plates and subjected to *M. stenopetala*, *C. citrinus* and *A. sativum* aqueous extracts at various concentrations ranging from 5mg/ml to 0.5mg/ml at 25^{o}C. Aliquots of the parasites were transferred into 96 well micro titer plates and incubated further at 27^{o}C in 5% CO_2 for 24 hours and then 200μl of the test single aqueous extracts at serially diluted concentrations or fixed ratios ranging from 9:1 to 1:9 of the blends, were added. The control wells contained Schneider's insect medium alone. The plates were then incubated further at 27^{o}C for 48 hours. Ten micro liters of MTT reagent was added into each well and incubated further for 4 hours. The medium and MTT were aspirated off the wells. Then in each well, 100μl of Dimethyl sulfoxide (DMSO) was added and the plates shaken for 5 minutes. Absorbance was

read at 562 nm, and percentage promastigotes' viability (%) was determined as described by Mosmann (1983) using the formula: Viable promastigotes (%) = (*AT* – *AB*) / (*AC*) × 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.

In vitro drug interaction experiments

Corresponding IC_{50} values were determined for each single extract and in combination (Sixthmith *et al*., 1984). The degree of synergy was evaluated according to the method described by Berenbaum (1978). Sum of fractional inhibition concentration (SFIC) abbreviated as K was calculated using the formula: $A_c/A_e + B_c/B_e = K$, where A_c and B_c were equally effective concentrations (IC_{50}) when used in combinations, and A_e and B_e were the equally effective concentrations (IC_{50}) when used alone. In this system, SFIC < 1 denoted synergism, 1 ≤ SFIC < 2 denoted additive interaction, while SFIC ≥ 2 denoted antagonism (Gupta *et al*., 2002). SFIC values (degrees of synergy) were calculated for the blends of aqueous extracts in all the fixed ratios (9:1 to 1:9).

In vitro anti amastigote assay

The anti-amastigote assay was carried out as described by Delorenzi *et al*. (2009). Ten milliliters of sterile cold PBS was injected into the peritoneum of anaesthetized and disinfected clean BALB/c mice. The retrieved PBS that contained peritoneal macrophages was centrifuged at 2,000 rpm for 10 minutes. The macrophages obtained were then adsorbed in sterile 24-well micro titer plates for 4 hours at 37°C in 5% CO_2. Adherent macrophages were then infected with promastigotes and incubated further for 4 hours followed by washing off the free promastigotes with sterile PBS. The infected macrophages were incubated further for 24 hours in RPMI 1640 culture medium. The infected macrophages were then treated once with individual aqueous extracts of *M. stenopetala* (A), *C. citrinus* (B), or *A. sativum* (C) or their blends which included *M. stenopetala* and *C. citrinus* (AB), *C. citrinus* and *A. staivum* (BC) and *M. stenopetala* and *A. sativum* (AC) at a fixed ratio of 1:1 and MIC based concentrations. Pentostam, liposomal amphotericin B and RPMI medium were used as controls. The medium, test extracts and control drugs were replenished daily for 3 days. After 5 days, the macrophages were washed with PBS at 37°C, fixed in methanol and stained with 10% Giemsa. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and the results was expressed as infection rate (IR) and multiplication index (MI) as described by Berman and Lee (1984).

In vivo infection and treatment of BALB/c mice

The infection of BALB/c mice with *L. major* was carried out as described by Gamboa-Leon *et al*. (2007). Each experimental group comprised of 5 BALB/c mice of the same sex. The left hind footpads of the mice were subcutaneously inoculated with 1×10^6 stationary phase infective metacyclic promastigotes of *L. major* in 40*µ*l sterile PBS. Lesions development was monitored weekly by measuring the thickness of infected left hind foot pad and comparing it with that of non infected right hind foot pad using a vernier caliper. Lesion size was expressed as the difference between the infected and the contralateral non-infected footpad. Treatment with combined aqueous extracts at fixed ratio of 1:1 or control drugs commenced one month post infection. Treatment was done orally using a cannula and intra-peritoneally using fine 1ml, 30 gauge Insulin needles (BD Micro-Fine Plus®, USA) at a dose of 20 mg/kg daily. Similarly, positive control groups of mice were treated with pentostam and liposomal amphotericin B which were administered intra-peritoneally at standard dose of 20 mg/kg per day.

Estimation of parasite burden in infected BALB/c mice

After 4 weeks of treatment, the mice were subjected to 100*µ*l pentobarbitone sodium (Sagatal®) and sacrificed. At necropsy, the spleens were weighed and their impression smears made as described by Chulay and Bryceson (1983). The impression smears were

fixed in methanol and stained with Giemsa. The slides were examined under a microscope to enumerate the number of amastigotes per 1000 nucleated cells. The relative and total numbers of amastigotes in infected mice spleens were estimated by calculating the Leishman-Donovani Unit (LDU) and total Leishman-Donovani Unit (number of amastigotes /1000 spleen nuclei × spleen weight in mg × (2×10^5) as described by Bradley and Kirkley (1977).

Statistical analysis

Data was analyzed using SPSS version 17.0 for windows at 5% level of significance. One way ANOVA (F test) was used to compare promastigotes viability (%) after being subjected to different concentrations or ratios of test aqueous extracts (A, B, and C) and control drugs. Other variables compared using F test were infection rates (IRs), multiplication indices (MIs) of amastigotes in peritoneal macrophages and lesion sizes in infected and treated BALB/c mice. Multiple comparisons of the individual treatments were done using Tukey's HSD *post hoc* test.

Results

Plant extracts yields

The aqueous yields of *M. stenopetala*, *C. citrinus*, and *A. sativum*, were 3.2 g (6.40 %), 8.79g (17.58 %), and 9.29g (18.59 %) respectively.

Minimum inhibitory concentrations (MIC) of the promastigotes

The MICs of single aqueous extracts *M. stenopetala* (A), *C. citrinus* (B) and *A. sativum* (C) against *L. major* promastigotes were 3mg/ml, 5mg/ml and 5mg/ml respectively. In comparison, the MICs of pentostam and liposomal amphotericin B were 12.50μg/ml and 6.25μg/ml respectively. The Schneider's insect medium supported the survival of *L. major* promastigotes maximum (Table 1). The survival of *L. major* promastigotes after treatment with fixed ratios of combined aqueous extracts that ranged from 9:1 to 1:9 and at concentrations that were based on MICs of the individual extracts, was relatively low at ratio 1:1 (Table 1).

Table 1: Survival of the *L. major* promastigotes in varying concentrations of blends of aqueous extracts as observed under a light microscope.

		Ratio of the two extracts based on MIC						
Test extracts and controls	**Code**	**9:1**	**8:2**	**6:4**	**1:1**	**4:6**	**2:8**	**1:9**
M. stenopetala: *C. citrinus*	A: B	+	+	+	-[a]	++	++	++
M. stenopetala: *A. sativum*	A: C	+	++	+++	++	++++	++++	++++
C. citrinus: *A. sativum*	B: C	++	++	++	++	++	++	+++
		Concentrations of the standard drugs (μg/ml)						
+ve controls:			100	50	25	12.5	6.25	3.125
Pentostam			-	-	-	-	+	++
Liposomal Amphotericin B			-	-	-	-	-	+
-ve control								
Schneider's Insect Medium			++++[b]	++++	++++	++++	++++	++++

-[a] represented absence of detectable and live promastigotes; ++++[b] represented maximum survival (density) of the *L. major* promastigotes.

IC_{50} values of the test extracts and viability of the promastigotes

The single aqueous extracts of *M. stenopetala*, *C. citrinus*, and *A. sativum* had IC_{50} that ranged from 297μg/ml to 575μg/ml against promastigotes. Under similar experimental conditions, pentostam and liposomal amphotericin B had IC_{50} values of 0.26μg/ml and 0.82μg/ml respectively and therefore very little quantities of these drugs were needed to inhibit *L. major* promastigotes *in vitro* (Table 2). Pentostam was more toxic as indicated by a high $-\log_{10}IC_{50}$ of

0.59. For blends AB (*M. stenopetala* and *C. citrinus*), and BC (*C. citrinus* and *A. sativum*) , the most active ratio was 1:1 which corresponded to *in vitro L. major* promastigotes viabilities of 33.82% and 60.74% respectively (Tables 2 and Figure 1). Blend AC (*M. stenopetala* and *A. sativum*) was most active at 1:9 with promastigotes viability of 17.41% (Table 2). IC_{50} for the blends AB, BC, and AC ranged from 174μg/ml to 1314μg/ml (Table 2).

Table 2: The IC_{50} for single test aqueous extracts and their blends at fixed ratios and MICs for *L. major* promastigotes

Test extracts and controls	Code	MIC (*m*g/ml)	Active ratio	IC_{50} (μg/ml)	-$\log_{10}IC_{50}$ (pIC_{50} scale)	Viability[a] (%)
Single extracts:						
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
Combined extracts:						
M. stenopetala and *C. citrinus*	A: B	-	1:1	1314.00	-3.12	33.82
M. stenopetala and *A. sativum*	A: C	-	1:9	174.00	-2.24	17.41
C. citrinus and *A. sativum*	B: C	-	1:1	378.50	-2.58	60.74
Controls[b]:						
Pentostam		0.0125	-	0.26	0.59	18.41
Liposomal amphotericin B		0.00625	-	0.82	0.09	12.22
Schneider's Insect Medium		-	-	-	-	81.65

[a] = Concentration of the single extracts ranged between 5 to 0.5 mg/ml; [b] = the initial concentration of the control drugs was 100 μg/ml followed by serial dilution by a factor of 2.

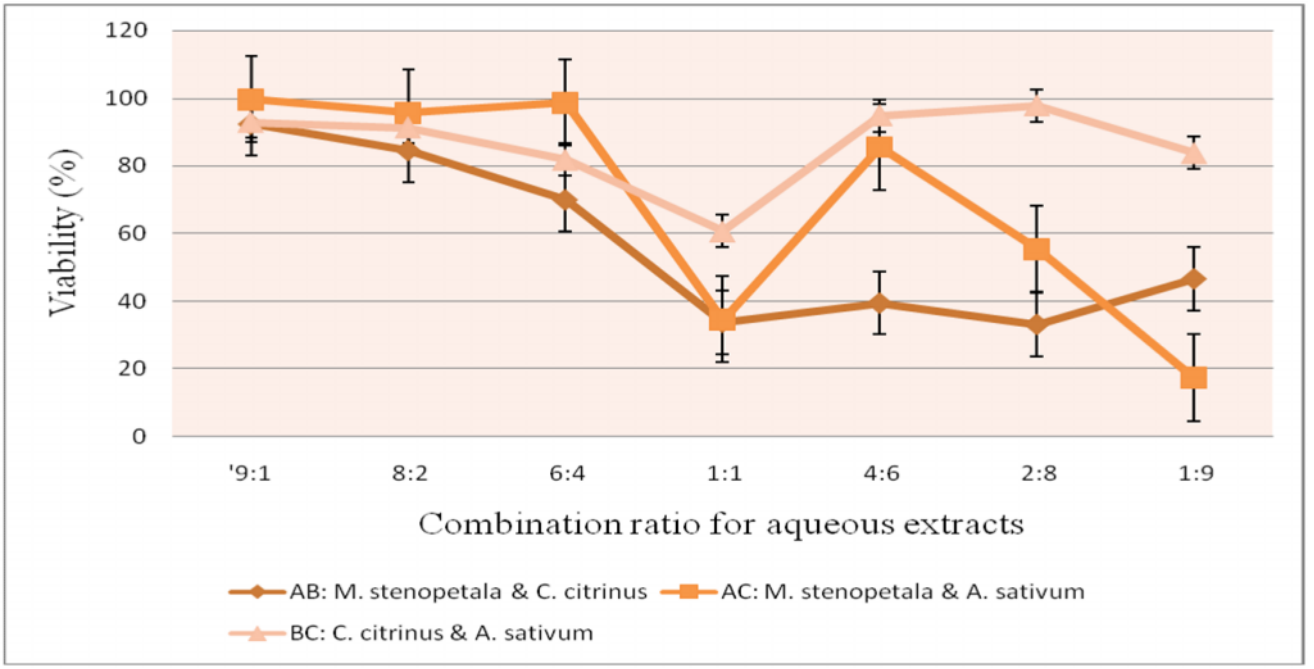

Figure 1: Viability (%) of *L. major* promastigotes when exposed to blends of two different aqueous extracts at fixed ratios *in vitro*.

In vitro drug interactions

Combined aqueous extracts of *M. stenopetala* and *C. citrinus* (AB) showed a marked additive interaction in ratios 8:2, 6:4, 4:6 and 1:9 as well as synergistic interaction in ratios 5:5 and 2:8 and a weak antagonistic interaction at ratio 9:1 (Table 3). As the content of *C. citrinus* increased in blend AB, the interac-

tion tended to change from antagonistic to additive (Table 3). Strong synergistic interaction (at ratio 1:9) and additive interactions (at ratios 5:5 and 2:8) were noted for the blend of *M. stenopetala* and *A. sativum* (AC) (Table 3). Increase of *A. sativum* (C) proportion in blend AC, tended to shift the interaction from additive to synergistic. Blend of *C. citrinus* and *A. sativum* (BC) had a strong antagonistic interaction in majority of the ratios except at ratio 5:5 which had moderate additive interaction (Table 4).

Table 3: The SFIC values that represented interaction of aqueous extracts of *M. stenopetala* (A) with *C. citrinus* (B) and *A. sativum* (C) upon blending at fixed ratios

	Ratio (A: B and A: C)						
Plant extract	**90: 10**	**80: 20**	**60: 40**	**50: 50**	**40: 60**	**20: 80**	**10: 90**
A. sativum (C)	2.201[c]	1.854[b]	1.602[b]	0.976[a]	1.075[b]	0.966[a]	1.198[b]
C. citrinus (B)	3.176[c]	3.857[c]	3.798[c]	1.180[b]	2.993[c]	1.606[b]	0.686[a]

The figures in the table are SFIC values, [a] = synergistic (SFIC < 1); [b] = additive (1 < SFIC < 2); [c] = antagonistic (SFIC ≥ 2). The mean SFIC values were derived from average absorbance and viability values.

Table 4: The SFIC values representing interaction of aqueous extracts of *C. citrinus* (B) with *A. sativum* (C) upon blending at fixed ratios.

	Ratio (B: C)						
Plant extract	**90: 10**	**80: 20**	**60: 40**	**50: 50**	**40: 60**	**20: 80**	**10: 90**
A. sativum (C)	3.039[c]	2.995[c]	2.730[c]	1.444[b]	3.094[c]	3.175[c]	2.783[c]

The figures in the table are SFIC values, [a] = synergistic (SFIC < 1); [b] = additive (1 < SFIC < 2); [c] = antagonistic (SFIC ≥ 2). The mean SFIC values were derived from average absorbance and viability values.

Effect of single and combined aqueous extracts on L. major amastigotes in vitro

At a concentration of 125μg/ml, the aqueous extracts of *M. stenopetala* (A), *C. citrinus* (B) and *A. sativum* (C) supported *in vitro* amastigotes infection rates (IR) of 58%, 75%, and 51% respectively compared to liposomal amphotericin B and pentostam which had IRs of 6% and 14% respectively at concentrations of 50μg/ml. RPMI medium supported the growth of *L. major* amastigotes effectively as indicated by a high IR of 84.67± 2.96 %. The difference of the mean IRs for single aqueous extracts and those for control drugs was significant ($P < 0.05$). The multiplication indices (MIs) for liposomal amphotericin B and pentostam were 8.60% and 11.54% respectively at concentration of 50μg/ml and the difference was not significant ($P = 0.155$). The blend of *M. stenopetala* and *A. sativum* (AC) at ratio 1:1 had an IR of 71% and an MI of 48.20% and this was comparable to that of pentostam with IR and MI of 67% and 47.51% respectively at concentration of 12.50μg/ml. A blend of *C. citrinus* and *A. sativum* (BC) and a blend of *M. stenopetala* and *C. citrinus* (AB) were associated with IRs ranging from 81% to 86% and MIs ranging from 65.84% to 72.40% which were closely comparable to that of pentostam with IR of 81% and MI of 69.46% at a concentration of 6.25μg/ml.

Effect of combined aqueous extracts on lesion sizes in BALB/c mice

Orally administered blend of *M. stenopetala* and *C. citrinus* (AB) aqueous extracts (1:1 ratio) into *L. major* infected BALB/c mice, caused a reduction of foot pad lesion size, that was not significantly different from that of infected mice treated with pentostam or liposomal amphotericin B ($P > 0.05$). However, a blend of aqueous extracts of *C. citrinus* and *A. sativum* (BC) reduced the footpad lesion size significantly ($P < 0.05$), when compared to PBS, pentostam, and liposomal amphotericin B controls (Figure 2). On the contrary, a blend of aqueous extracts of *M. stenopetala* and *A. sativum* (AC) caused non significant reduction ($P > 0.05$) of foot pad lesions when compared with oral PBS ($P = 0.714$), pentostam ($P = 1.000$) and liposomal amphotericin B ($P = 0.998$) controls. Similarly, the lesion sizes in BALB/c mice treated with the blends AB, BC, and AC that were administered intraperitoneally (ip), were significantly different ($F = 4.806$, $P < 0.05$) from those in mice treated with liposomal amphotericin B, pentostam, PBS (ip) and PBS (oral). Tukey's *post hoc* test, however, showed that both AB (ip) and BC (ip) differed significantly ($P < 0.05$) from both PBS (oral) and PBS (ip) but not significantly different ($P > 0.05$) from both pentostam and liposomal amphotericin B. AC (ip) was not significantly different ($P > 0.05$) from all the control drugs.

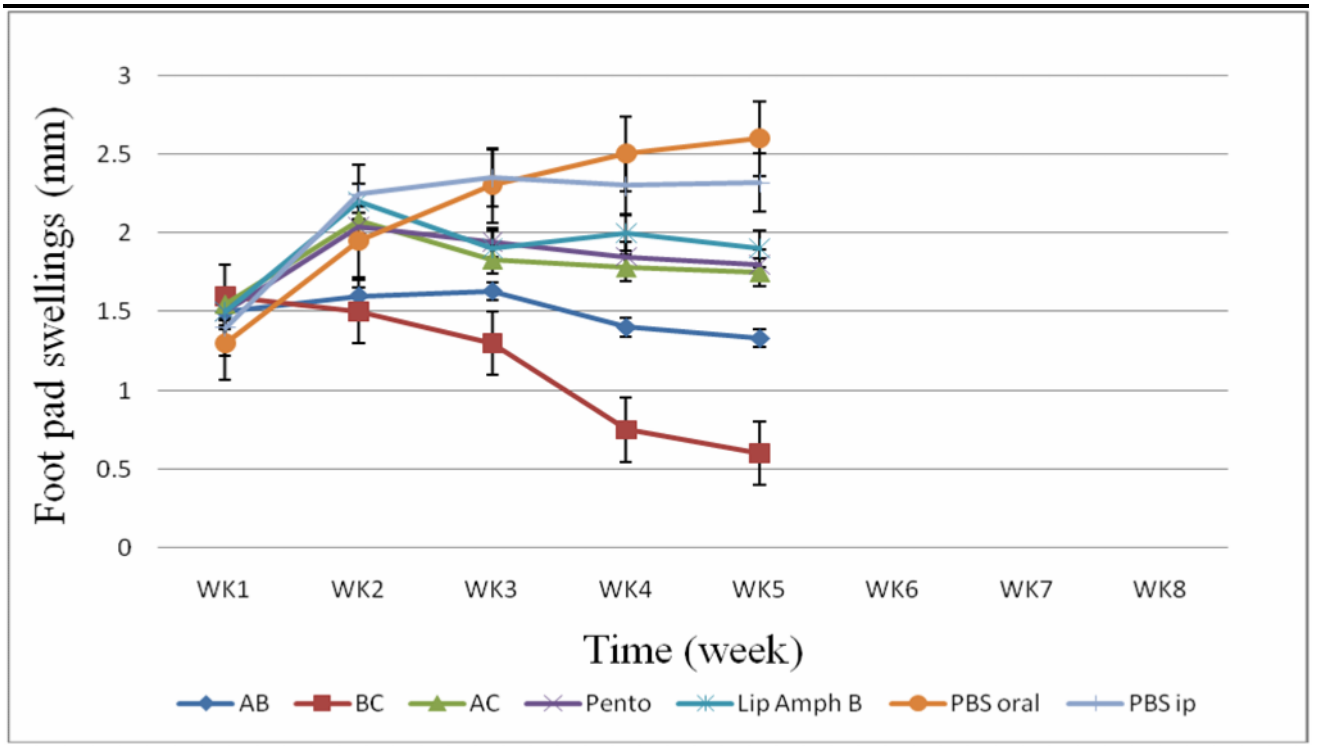

Figure 2: The effect of oral treatment in *L. major* infected BALB/c mice with combined aqueous extracts (1:1 ratio) of *M. stenopetala* and *C. citrinus* (AB); *C. citrinus* and *A. sativum* (BC); *M. stenopetala* and *A. sativum* (AC) and the controls pentostam (ip), liposomal amphotericin B (ip) and phosphate buffered saline (PBS**).**

Table 5: The average spleen index ± SE, LDU ± SE and total LDU ± SE for *L. major* infected BALB/C mice followed by treatment with combined aqueous extracts (blends)or control drugs or PBS

Treatment	Route	Spleen Index (%)	LDU	Total LDU (× 1000)	% parasite reduction[a]
Blends (ratio 1:1):					
AB	oral	0.62 ± 0.07	0.56 ± 0.35	11.49 ± 6.84	6.51
	ip[b]	0.70 ± 0.09	0.33 ± 0.18	7.83 ± 3.24	36.28
BC	oral	0.67 ± 0.00	0.26 ± 0.03	6.35 ± 0.66	48.33
	ip	0.50 ± 0.06	0.18 ± 0.06	3.19 ± 1.16	74.04
AC	oral	0.59 ± 0.06	0.20 ± 0.02	4.80 ± 0.95	60.94
	ip	0.46 ± 0.04	0.33 ± 0.06	7.22 ± 0.66	41.25
Controls:					
Pentostam	ip	0.73 ± 0.19	0.18 ± 0.08	4.13 ± 1.10	66.40
Lip amhotericin 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	28.88
PBS	oral	0.56 ± 0.06	0.61 ± 0.22	12.29 ± 4.49	00.00

[a] The % was calculated in reference to total LDU for PBS oral which was taken as maximum (100%) parasite burden; ip[b] = intra peritoneal; AB = *M. stenopetala* and *C. citrinus*; BC = *C. citrinus* and *A. sativum*; AC = *M. stenopetala* and *A. sativum*; PBS = phosphate buffered saline.

Parasite burden in the spleens of L. major infected BALB/c mice

Orally administered blends BC and AC reduced the parasite burden in BALB/c mice spleens by 48.33% and 60.94% which corre-

sponded to total LDUs of 6.35±0.66 and 4.80±0.95 respectively. Similarly, oral blend AB reduced the parasite burden by 6.5% with a total LDU of 11.49±6.84 (Table 5). It was noted that the higher the total LDU values, the lower was the percent parasite reduction. Intra peritoneally administered blends AB, AC and BC reduced the spleen parasite burden by a range of 36.28% to 74.02% with total LDUs of 3.19% to 7.83% (Table 6). The efficacy of combined aqueous extracts BC (ip) and AC (oral) were comparable to the efficacy of pentostam and liposomal amphotericin B. There was no significant difference (P = 0.098) between the total LDUs in BALB/c mice treated orally and those treated intra peritoneally for blends AB, BC, AC and control PBS. Parasite reduction (%) in BALB/c mice treated intra-peritoneally was relatively higher than those treated orally for blends AB and BC (Table 5).

Discussion

Combination therapy as a protocol of treatment is often adopted for many infectious diseases particularly in cases where the infectious agent fails to respond to the monotherapeutic regimen. Several studies on leishmaniasis, indicate that a combination therapy of antileishmanial drugs improves their efficacy and reduces resistance, dosage and toxicity levels of the drugs (Nyakundi *et al*., 1994; Melaku *et al*., 2007; Sundar *et al*., 2008). Following combination of drugs, synergistic, additive or antagonistic effects may ensue. In the present study, blends of *M. stenopetala* and *C. citrinus* (AB), *M. stenopetala* and *A. sativum* (AC) and *C. citrinus* and *A. sativum* (BC) aqueous crude extracts at various fixed ratios showed marked synergistic and additive interactions that were associated with inhibition of *L. major* promastigotes survival *in vitro*. According to Tahany (2010), additive and synergistic effects are advantageous while antagonistic effects are disadvantageous. These observations were in line with those of Gathirwa *et al*. (2008), who reported that when malaria herbal extracts were combined, those that showed marked synergistic and additive interactions, were associated with a high anti-plasmodial activity.

Reliable leishmanicidal activity must be complemented with use of intracellular amastigotes in macrophages (Chan-Bacab and Pena-Rodriguez, 2001). Since the infection rates of *L. major* amastigotes in macrophages treated with the single test extracts were significantly lower than those treated with negative control, it was deduced that the extracts had inhibitory effect on amastigotes. The present study established that the axenic amastigotes were more sensitive to a lower concentration of the blends of aqueous crude extracts (AB, AC, and BC) than promastigotes which required higher dose of the crude extracts. This was in line with a previous observation made by Abdul-rahman *et al*., 2009. According to Berman and Wyler (1980), the sensitivity of amastigotes and promastigotes to antileishmanial agents tend to differ. The variation in sensitivity is probably inherent or attributable to immune mechanisms that are taking place in the macrophage in which the amastigotes are engulfed in the phagolysosome (Berman and Wyler, 1980). The efficacy of blend AC with an inhibition rate of 16% against amastigotes in peritoneal macrophages was higher than those of blends AB and BC which were at < 4%. These observations were in line with a report by Yousefi *et al*. (2009), in which a combination of *Alkana tincturia* and *Peganum harmala* crude extracts in a ratio of 1:1 (10μg :10μg) had a better *in vitro* effect at low dose against *L. major* as compared to the effect of separated extracts.

The blends of two aqueous extracts in the ratio 1:1 that had *C. citrinus* extracts (B) incorporated tended to be more effective in reducing the foot pad lesion sizes in *L. major* infected BALB/c mice. The synergistic and additive interactions in blends BC, AB, and AC at ratio of 1:1 and MIC based concentrations may have contributed to their efficacy in reducing the foot pad lesion sizes. According to Gharavi *et al*. (2011), a combination of aqueous *A. sativum* and glucantime was observed to be much more effective in decreasing lesion size than either glucantime or aqueous *A. sativum* alone and that the combination triggered a Th-1 type of immune response. Leishmaniases tend to be endemic in poverty stricken tropical areas where the residents are likely to address it through herbal therapy that may comprise of several

herbs administered concurrently, unfortunately without a clear dose for each herb. The present study shows that the efficacy of blends of two different extracts at equal ratio and at MIC based concentrations was good in limiting the survival of *L. major*.

Blends of *C. citrinus* and *A. sativum* (BC-ip) and *M. stenopetala* and *A. sativum* (AC-oral) were efficacious as indicated by low total LDU values and parasite reduction rates of 74.04% and 60.94% respectively as compared to pentostam (66.40%) and liposomal amphotericin B (60.62%) under the same experimental conditions. This observation was in line with a recent report by Makwali *et al*. (2012) in which a combination therapy comprising of trifluralin and acriflavine compounds were associated with low LDU levels in treated BALB/c mice. The additive interactions of the individual extracts in the blends (BC-ip and AC-oral) may have contributed to their activeness in inhibiting parasites in the spleens of BALB/c mice. The percentage parasite reduction rates of the individual aqueous extracts including oral *M. stenopetala* (A), oral *C. citrinus* (B), ip *C. citrinus* (B), and oral *A. sativum* (C) in BALB/c mice spleens were 66.96%, 82.99%, 75.18%, 60.37% respectively compared to an average of 63.51% for pentostam and liposomal amphotericin B. These plant extracts therefore possessed antileishmanial activity even though they were relatively less toxic (high IC_{50} values) when compared to pentostam and liposomal amphotericin B. Their low toxicity levels explained why garlic and African moringa are used as foods in many parts of the world while bottle brush extracts are used as a hot drink popularly referred to as the 'tea' in Jamaica for gastro enteritis, diarrhea and skin infections. These observations from the current study were in line with several previous reports. For instance, antileishmanial potency of *M. stenopetala* extracts has been documented (Nordos *et al*. 2011). Majority of drugs test studies on *C. citrinus* species have concentrated on antimicrobial activity. Methanolic and aqueous extracts of *C. citrinus* have been reported to be effective inhibitors of a broad spectrum of bacteria *in vitro* (Cock, 2008; Delahaye *et al*., 2009; Oyedeji *et al*., 2009; Seyydnejad *et al*., 2010; Abdelhardy and Aly, 2012). The present study adds to this list by showing that *C. citrinus* aqueous crude extracts have anti protozoan activity also. *A. sativum* extracts have been documented as having potent anti leishmanial compounds which enhance the phagocytic and killing activities of host macrophages (Ghazanfari *et al*., 2006; Gamboa-Leon *et al*., 2007). Furthermore, aqueous garlic extracts have the ability to stimulate the interferon gamma (IFN-γ) genes in *L. major* infected macrophages hence promoting the destruction of engulfed amastigotes (Gharavi *et al*., 2011). The current study agrees with these reports on garlic since the blends that possessed garlic extracts were active against *L. major*.

Conclusion

Blends of crude aqueous extracts of *M. stenopetala, C. citrinus,* and *A. sativum* possess *in vitro* and *in vivo* antileishmanial activity against *L. major* promastigotes and amastigotes. The blending ratios of crude extracts that exhibited synergistic and additive interactions were associated with low viabilities of the parasites *in vitro*. An orally administered blend of crude aqueous extracts of *C. citrinus* and *A. sativum* (1:1 ratio) had the highest efficacy in reducing the footpad lesion size in *L. major* infected BALB/c mice when compared to others blends and controls in the study. Research is underway to identify the most active fractions in crude extracts of *M. stenopetala, C. citrinus*, and *A. sativum* against *L. major* and to further test their efficacy when blended in fixed ratios.

Acknowledgements: The authors thank the Director of Center of Biotechnology and Research Development (CBRD) at Kenya Medical Research Institute (KEMRI), Nairobi and all the researchers at the center for their technical advice and availing equipments. We are grateful to Simeon Mathenge of University of Nairobi herbarium for identification of the study plants. We also thank Mr. David Muchina for collecting plant materials from Baringo County in Kenya. We appreciate Mr Dalmas Odiri and Charles Muthaura of the Center of Traditional Medicine and Drug Research (CTMDR) at KEMRI for their technical

assistance. This work formed part of the requirements for the degree of PhD of Kenyatta University, Kenya, for the first author.

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