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In vitro and *in vivo* efficacy of combination therapy using *Allium sativum* and *Aloe secundiflora* against *Leishmania major* infected BALB/c mice

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Abstract
Allium sativum and *Aloe secundiflora* water extracts have antileishmanial activities. In the present study, the efficacy of combination therapy using *A. sativum* and *A. secundiflora* against *L. major* was studied both intraperitoneally and orally. The standard drug pentostam and phosphate buffered saline were used as positive and negative controls. T-test and ANOVA were used for data analysis and P-value of < 0.05 was considered significant. Plant materials were dried, ground soaked in water at 80 ºC for 1 hour, filtered then freeze dried. The minimum inhibitory concentrations of aqueous extracts of *A. secundiflora* and *A. sativum* were 2000 μg/ml and 5000 μg/ml and IC_{50} were 467.09μg/ml and 457.88μg/ml respectively. The IC_{50} for their combination at ratio (1:1) was 391.79 μg/ml as compared to minimum inhibitory concentrations of 12.5μg/ml and IC_{50} of 108.58μg/ml for pentostam. The combination therapy had Infection rate of 17% and multiplication index of 48.65% compared to pentostam (IR=21% and MI=11.64%). The combination therapy reduced the footpad lesion size significantly ($P<0.05$) like the pentostam control drug and no significant nitric oxide stimulated. The oral and intraperitoneal combination treatment reduced spleen amastigotes in mice by 55.48% and 64.13% corresponding to total LDUs of 18.23±0.90and 14.69±1.33 respectively compared to pentostam 94.58% and LDU of 2.22±0.13. The combination therapy was less toxic, effective against *L. major* parasite, reduced lesion size significantly, reduced spleen parasite load significantly but did not prevent visceralization.

Keywords: *Allium sativum, Aloe secundiflora, Leishmania major,* BALB/c Mice

Introduction
Leishmaniases are one of the major tropical neglected zoonotic parasitic disease caused by obligate intracellular protozoan parasites of the genus *Leishmania* [1]. The infection is transmitted through bites by infected female sandflies of the genus *Phlebotomus* in the old world and *Lutzomyia* in new world [2].
The global burden of leishmaniasis 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 [3]. The leishmaniases are associated with poverty as treatment is expensive and unaffordable [4]. Leishmaniasis is characterized by three forms: visceral leishmaniasis (VL), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL) [5]. The type of the disease expressed depends on type of *Leishmania* species. Clinical manifestations depend on the host's specific immune responses to parasite antigens and species [6]. Several studies have shown that natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans. There is need to explore maximally these sources because they are likely to lower the high cost of treatment, reduce resistance of drugs and reduce environmental pollution. This study aims to analyze the combining effect of crude extracts of *A. sativum* and *A. secundiflora* on *L. major* which causes leishmaniasis. *A. sativum* contains organosulfur compounds that have been attributed to medicinal properties present in the bulbs [7]. *Aloe secundiflora* contains several major groups of chemical compounds namely tannins, saponins, alkaloids, cardiac glycosides, flavonoids and terpenes [8].

Materials and methods
Source of the plant extracts and extraction process: The A. *sativum* bulbs were purchased from Marikiti market, Nairobi Kenya. Leaves of *A. secundiflora* were harvested from a

selected homestead in Ruai, Nairobi County, Kenya. The cloves of *A. sativum* were removed from the bulbs then sliced into small pieces and thereafter dried for 17 days at room temperature. Leaves of *A. secundiflora* were also sliced into small pieces and dried for 14 days at room temperature. They were separately ground into fine powder. Extraction was done as follows; 200g of the dried ground plant material was dissolved in 1000ml of distilled water and placed in a water bath at 75 ^{0}C for 1.5 hours. The extract was then filtered using Whatman NO. 1 filter papers and the filtrate was freeze dried and weighed [9]. The final extracts of *A. sativum* and *A. secundiflora* were coded as AS & AF respectively and then were stored at 4ºC until analysis.

Preparation of the test drugs: Stock solutions of 1mg/ml concentration of crude extracts were prepared by weighing 10mg of single test extracts and dissolved in 10ml of PBS for each extract separately for *in vitro* anti-leishmanial assays then filtered through 0.22μm filter flasks in a laminar flow hood. The combination drug (C) was made by weighing 2mg of *A. secundiflora* and 5mg of *A. sativum* both were dissolved in 1ml of PBS for *in vivo* work. The stock solutions were stored at 4 ºC and retrieved when used for *in vitro* and *in vivo* assays.

Experimental animals: Inbred eight weeks old BALB/c male mice were used. They were housed at the animal house at 25 ^{0}C and were fed on mice pencils which are a commercial diet and were given tap water and *libitum*. The mice were used for *in vivo* assays (amastigote and promastigote assays).

Cultivation of *Leishmania* major parasites: The *Leishmania major* strain (IDUB/KE/94=NLB-144) was obtained from CBRD, *Leishmania* laboratory section where it had been cryopreserved in liquid nitrogen. The parasites were grown to stationary phase at 25ºC for 15 minutes at 4 ºC in Schneider's *Drosophila* medium supplemented with 20% heat inactivated fetal bovine serum (FBS)-HYCLONE® USA), 100 U/ml penicillin and 500μg/ml streptomycin [10], and 250μg/ml 5-fluorocytosine arabinoside [11]. The stationary-phase metacyclic stage promastigotes were then harvested by centrifugation at 1500 rpm. The metacyclic promastigotes were then used for the *in vitro* and *in vivo* assays.

Cytotoxicity Assay: The assay was used to test the cytotoxicity of the individual extracts and combination therapy against Vero cells. The Vero cells were grown in minimum essential medium (MEM) supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100μg/ml) in 25ml cell culture flasks incubated at 37 ºC in a humidified 5% CO_2atmosphere for 24 hours. The Vero cells were harvested by trypsinization and pooled in 50 ml centrifuge tubes from which 100μl of the cell suspension were put into 2 wells of rows A-H in a 96-well flat bottomed microtitre plate at a concentration of 1×10^6 cells per ml of the culture medium per well and incubated at 37 ºC in 5% Co_2 for the cells to attach. The MEM was gently aspirated off and 150 µl of the highest concentration (1000µg/ml) of the test extracts (AS, AF and AS/AF) was added and serially diluted by a factor of 3 up to a concentration of 1.37μg/ml at wells of row B. The microtitre plates containing the Vero cells and test extracts were further incubated at 37 ^{0}C for 48 hours in a humidified 5% CO_2 atmosphere. The control wells comprised of medium with Vero cells while the blank wells had medium alone. 10μl of MTT reagent was added into each 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 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 562 nm. Cell viability was calculated at each concentration using the formula below. The IC_{50} values of the extracts were determined automatically using the Chemosen software program [12].

Cell viability (%) = Average absorbance in duplicate drug wells - average blank wells x 100 Average absorbance in control wells

Evaluation of Minimum inhibitory Concentration (MIC): The *L. major* promastigotes at a concentration of $1x10^6$ per ml were grown in Schneider's insect medium (SIM) in 24 well micro titer plate containing the test aqueous extracts (AF, AS and C) [13] in concentrations that ranged between 5mg/ml and 0.5mg/ml. Survival of the promastigotes upon exposure to five different fixed ratios (1:1, 1:2, 3:1, 3:1, 2:1) for blends of AF/AS was determined. The combination ratio that supported the least survival of promastigotes growth was noted [14].

Anti-Amastigote Assay: BALB/c mice was induced by injecting 2% starch intra peritoneally and left for 24 hours. The mouse was anaesthetized using 100μl pentobarbitone sodium (Sagatal®). The body surface of the mouse was disinfected with 70% ethanol after which it was torn dorso-ventrally to expose the peritoneum. Ten milliliters of sterile cold PBS was injected into the peritoneum. After injection, the peritoneum was gently massaged for 2 minutes to dislodge and release macrophages into the PBS. The peritoneal macrophages were then harvested by withdrawing the PBS. The PBS containing the macrophages was washed by centrifuging at 2,000 g for 10 minutes and the pellet obtained was re-suspended in RPMI culture medium. The macrophages were adsorbed in 24-well plates for 4 hours at 37 ºC in 5% CO_2. Non-adherent cells were washed with cold sterile PBS and the adherent macrophages were incubated overnight in RPMI culture medium. Adherent macrophages were then infected with *L. major* promastigotes and were further incubated at 37 ºC in 5% CO_2 for 4 hours after which they were washed with sterile PBS to remove the free promastigotes, that were not engulfed by the macrophages. This was followed by incubation of the preparation for 24 hours in RPMI 1640 culture medium. The infected macrophages were then treated with combined aqueous extracts of *A. secundiflora*/*A. sativum* (C) MIC based concentrations in fixed ratios of 1:1. Pentostam was used as a positive control drug to compare the parasite inhibition with that by the plant extracts. The medium and test extracts or drug were replenished daily for 3 days. After 5 days, the macrophages were washed with sterile PBS at 37 ºC, 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 count was expressed as infection rate (IR) and multiplication index (MI) as in the two formulae below [15, 16].

IR = Number of infected macrophages in 100 macrophages

$$MI = \frac{\text{Number of amastigotes in experimental culture/100 macrophages x 100}}{\text{Number of amastigotes in 100 control culture/100 macrophages}}$$

Determination of Nitric oxide production: Nitric Oxide (NO) release in macrophages culture was measured. BALB/c peritoneal macrophages were placed in each well in 96- well plates and allowed to adhere at 37 ºC in 5% CO2 in humidified atmosphere for 2 hours. Then RPMI-1640 with 10% FBS was added to the wells containing the cells and finally the test drugs (AF, AS, AF/AS) and controls were added and the mixtures were further incubated for 48 hours. 100 ml of the supernatants were collected for NO measurements [17]. The assay was done in triplicate. Measuring of NO was done by 60 ml of Greiss reagent A (1% Sulphanilamide in 1.2 M HCL) was added followed by 60ml of Greiss reagent B (0.35 N[1-naphthy] ethylenediamine). The plates were read at 540nm in the enzyme linked immunosorbent assay (ELISA) reader. Sodium nitrite in RPMI was used to construct a standard curve for each plate reading [18].

*Infection and treatment of BALB/c mice***:** Inoculations were done using fine 1ml, 30 gauge insulin needles, the thicknesses of hind footpads of the mice were measured using a reading 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. The right footpad was left as a contra lateral control. Lesions were left to develop for 4 weeks [16, 19]. After four weeks, treatment with test extracts and controls commenced after the lesions had developed.

Lesion size measurements: Lesions development and progression was monitored weekly by using a direct reading vernier caliper to measure the thickness of the infected left hind foot pad and comparing it with that of non-infected right hind foot pad. The infected footpads of all mice in different treatment groups were measured using a direct reading vernier caliper and lesion size calculations were done using the method as follows:

Lesion size = Size of infected footpad-Contra lateral uninfected footpad (mm) [19].

Determination of spleen parasite loads in splenic impression smears: After 5 weeks of treatment, mice were sacrificed by inoculating 60mg/kg body weight of penta barbitone sodium. Spleen impression smear were made on clean microscope slides. They were left to dry for 15 minutes followed by fixation using absolute methanol. The fixed slides were immersed in a freshly prepared 5% Giemsa stain solution for 20 minutes then flushed with tap water and left to dry. The slides were examined under a compound microscope for enumerating the number of amastigotes per 1000 host nuclei at a high power magnification. The relative and total numbers of parasites in the spleen, named Leishman-Donovan Units (LDU) and total LDU respectively were calculated according to the formula as follows:

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

Total LDU = LDU x organ weight x 2 x 10^5 [20].

Statistical analysis: Data were analyzed using Statistical package for social sciences (SPSS) software programme utilizing student T-test (paired and one sample) and one way analysis of variance (ANOVA) with Tukey and Games-Howell test statistic as *Post hoc* where applicable. Descriptive statistics were used where appropriate. A p-value of less than or equal to 0.05 was considered significant. Data were organized into tables, line graphs or bar graphs.

Ethical considerations: Approval for the study was obtained from Kenya Medical Research Institute (KEMRI) Ethical Review Committee.

Results

Cytotoxicity and minimum inhibition concentration (MIC): Cytotoxicity of the aqueous single plant extracts on Vero cells were 467.09µg/ml for *A. secundiflora* and 540.14µg/ml for *A. sativum.* The combination drug showed a cytotoxicity level of 391.79µg/ml. The combination therapy showed high toxicity of 391.79µg/ml compared to the single plant extracts. One sample t-test showed that the difference was significant (t = 4.008, P = 0.028). The cell viability (%) increased with an increase in IC_{50} (low cytotoxicity) For instance, Vero cells treated with the slightly toxic combination AF/AS, had the lower viability of 90.9% compared to the less toxic extract of *A. secundiflora* with a viability of 98.15% and the least toxic *A. sativum* 99.4%. When promastigotes were exposed to different concentrations of single plant extracts, combination of the extracts and the control pentostam drug, *A. secundiflora* (AF) showed MIC of 2000 μg/ml or 2 mg/ml and *A. sativum* (AS) inhibited the survival of *L. major* promastigotes *in vitro* at concentration of 5000 μg/ml or 5 mg/ml. The combination of *A. secundiflora and A. sativum* had a minimum growth of promastigotes at ratio of 1:1. In comparison pentostam (+ve control) was able to inhibit the growth of *L. major* promastigotes *in vitro* at a concentration of 12.5μg/ml.

Table 1: IC50 (µg/ml) and the Minimum Inhibition Concentration

Drug	*A. secundiflora*	*A. sativum*	*A.sec/A.sat* (1:1)	Pentostam
IC_{50} (µg/ml)	467.09	457.88	540.14	108.88
MIC (µg/ml)	2000	5000	1:1(2000:5000)	12.5

Anti-Amastigote assay: This was done to test the efficacy of test extracts against amastigotes in the macrophages and was expressed in terms of infection rate (IR) and multiplication index (MI). The extent of the infection was determined by the number of infected macrophages and the number of amastigotes per 100 macrophages. Pentostam inhibited the *in vitro* survival of *L. major* amastigotes in BALB/c mice peritoneal macrophages more effectively at IR of 21% at a concentration of 50μg/ml which was very close to drug AF at IR of 23% at a concentration of 125μg/ml and combination drug (AF/AS)(1:1) at 17% while RPMI 1640 medium which had no drug in-cooperated supported the growth of *L. major* amastigotes in peritoneal macrophages more effectively and this was indicated by a high infection rate (IR) of 71.5± 2.12%. *A. sativum,* had IR of 53 at a concentration of 125μg/ml. One way ANOVA analysis of the IR% of AF, AS at 125μg/ml and C (1:1) compared to pentostam at concentration of 50μg/ml and RPMI showed no significant difference with $F_{(1,3)} = 1.369$ and P = 0.544. Comparison of IR% of the drugs at 31.25μg/ml (57%, 88%) and combination, C, (17%) with the controls, pentostam at 12.5μg/ml (37%) and RPMI (71.5%) showed no significant difference ($F_{(1, 3)} = 1.837$, P = 0.486). A Tukey *post hoc* test revealed that the multiplication index (%) of aqueous *A. sativum* (AS) was significantly different from that of RPMI (P = 0.015) and pentostam compared to RPMI was significantly different with a P = 0.003. The comparison

of multiplication indexes (%) of drug C and AS (*A. sativum*), drug C and pentostam was not significantly different (P = 0.07 and 0.631) respectively. However, comparison between drug C and RPMI was significantly different with a P= 0.020.

Stimulation of Nitric Oxide production: Peritoneal macrophages were incubated in RPMI-1640 medium for 48 hours in the presence or in absence of the plant extracts. The optical densities (OD) readings of the aqueous single plant extracts and their combination were all less than 0.10 that is they ranged between 0.030-0.092 (Table 4.6) at concentrations that ranged between 3.125 to 100µg/ml and this implied that, negligible amount of NO was produced (≤20µm of NO) as estimated from the standard nitrite curve. RPMI-1640 medium produced similar negligible levels of NO (negative control).

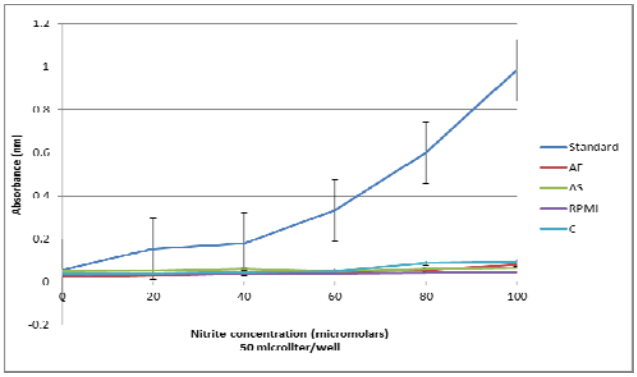

Fig 1: Nitric Oxide (µM) produced by BALB/c mice macrophages after exposure to different concentrations of single aqueous test extracts, combination and RPMI (100µg/ml serially diluted to 3.125µg/ml by a factor of 2).

Effects of combination therapy (C) on lesion sizes and body weights: When combined extracts of *A. secundiflora* and *A. sativum* (C) were administered intra-peritoneally and orally to treat *L. major* infected BALB/c mice, the foot pad lesion sizes tended to decrease for the first two weeks and remained constant for the last 3 weeks despite continued treatment. One way ANOVA (P<0.05) analysis showed there was a significant difference of the mean lesion sizes within and between the groups with F =9.613 and P = 0.0001. Post hoc tests of multiple comparisons (Games-Howell) showed there was a significant difference between combination (C) IP against PBS oral with a P=0.008 while there no significant difference combination (C) oral and PBS oral with a P=0.609. Pentostam too had a significant difference against PBS oral with a P=0.017.

Table 2: Average lesion size (mm) and Post hoc (Games-Howell) test P values against PBS oral in BALB/c mice infected with *L. major* treated with combination aqueous test extracts and controls administered orally or intra-peritoneal (ip) for a period of 5 weeks.

Mean f/pad swelling (mm) [a]							
Drug vs PBS oral	**Drugs Code Route**	**Wk1**	**Wk2**	**Wk3**	**Wk4**	**Wk5**	**sig. value**
A.sec/A. sat C	IP	0.24	0.23	0.16	0.13	0.13	0.008*
A.sec/ A. sat C	Oral	0.7	0.6	0.45	0.3	0.3	0.609
Controls:							
PBS	IP	0.38	0.68	1.12	1.02	0.79	0.725
PBS	Oral	0.29	0.57	0.79	0.79	0.79	N/A
Pentostam	IP	0.40	0.30	0.20	0.10	0.10	0.017*

Table 3: Showing Post hoc tests (Games-Howell) of multiple comparison of mean lesion size in different groups

Drugs compared	**Mean lesion difference**	**SE**	**P value**	**95% CI Lower Upper**	
C ip vs C oral	-0.292	0.122	0.158	-0.657	0.073
C ip vs PBS ip	-0.478	0.153	0.093	-1.035	0.079
C ip vs PBS oral	-0.326	0.127	0.171	-0.769	0.118
C ip vs Pento	0.100	0.099	0.844	-0.249	0.449
C oral vs PBS ip	-0.582*	0.139	0.042*	-1.138	-0.026
C oralvsPBS oral	0.430*	0.109	0.044*	-0.847	-0.013
C oral vs Pento	-0.004	0.075	1.000	-0.266	0.258
PBS oral vs Pento	0.578*	0.143	0.042*	0.026	1.130
PBS oral vs Pento	0.426*	0.115	0.047*	0.007	0.845

*shows significant difference between the two drugs, SE=Standard error of the mean, CI= Confidence Interval, ip= intraperitoneal.

The body weights for the combination therapy decreased for both ip and oral same to the negative control (PBS). The pentostam group the weights were unaffected. The t-test showed that there was no significant loss of body weights at P < 0.05 for infected BALB/c mice that were treated both orally or intraperitoneally with the combined drug as shown in table 4.8. However, the effect of the body weight is also affected by factors like nutrition, age, stress and the diseased state of the mice hence did not depend on the infection alone.

Table 4: Average body weights (g) of BALB/c mice infected with *L. major* and treated with combination test extracts and controls administered orally or intra-peritoneal (ip) over 5 weeks (weeks) period**.**

Paired t- test for the weight			**Mean body wgt (g)**		
Test drug	**Initial wgt ±SE**	**Final wgt ±SE**	**Difference (g)**	**Calculated t**	**p value**
Combination (IP)	21.67±2.03	20.67±2.33	-1.00	1.168	0.296 (ns)
Combination (oral)	30.25±1.16	29.38±0.82	-0.87	1.369	0.213 (ns)
PBS (IP)	20.07±0.52	19.33±0.33	-0.74	2.750	0.111(ns)
PBS (Oral)	21.00±0.56	18.67±0.33	-2.33	3.500	0.073(ns)
Pentostam (IP)	21.67±0.33	21.67±0.33	0.00	0.001	1.000(ns)

Key: ns= not significant, SE=Standard error of the mean.

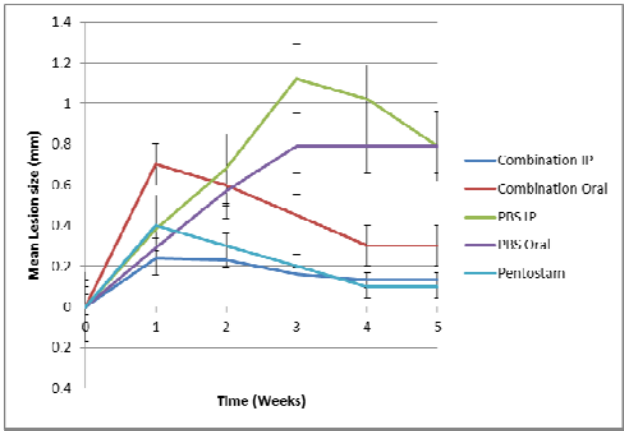

Fig 1: The foot pad swelling after treatment of *L. major* infected BALB/c mice with aqueous combination therapy (C) oral and intraperitoneally.

Estimation of number of *Leishmania* parasites in the infected BALB/c mice splenocytes: The mice were sacrificed, spleens weighed and their impression smears made and the number of amastigotes per 1000 nucleated splenocytes were counted. The spleen index (%), Leishman-Donovan Unit (LDU), total Leishman-Donovani Unit (total LDU) and percentage parasite reduction were determined. The ip combination therapy administration had the highest parasite reduction percentage of 64.13% as compared to oral combination therapy administration with 55.48%. Pentostam, the positive control, had the highest parasite reduction percentage of 94.58% as expected as it's the drug of choice for leishmaniasis. A small percentage was observed in the negative control (PBS). It was observed that the mice treated intraperitoneally had a higher % parasite reduction in their spleens (lower total LDU value) as compared to those treated orally. One sample t-test analysis of parasite reduction % showed a close significance with (t=2.531 and p=0.065).

Table 5: Average spleen weight, index, LDU, total LDU and % parasite reduction for groups of L. major infected BALB/c mice that were treated with combined test aqueous and controls

Drug/ Controls	Route	Ave spleen weight ±SE	Ave spleen Index (%) ±SE	Ave LDU ±SE	Ave total LDU ±SE(×1000)	% parasite reduction[a]
Combination ratio 1:1						
A. sec/A. sat	ip	0.125± 0.014	0.53 ± 0.07	0.59 ± 0.018	14.69 ± 1.33	64.13
A. sec/A. sat	oral	0.13± 0.01	0.50 ± 0.05	0.61 ± 0.03	18.23 ± 0.90	55.48
Control						
Pentostam	ip	0.10± 0.003	0.45 ± 0.19	0.11 ± 0.003	2.22 ± 0.13	94.58
PBS	ip	0.20± 0.010	1.01 ± 0.05	0.93 ± 0.012	37.15 ±1.09	9.28
PBS	oral	0.23± 0.020	1.10 ± 0.11	0.93 ± 0.001	40.95 ± 3.75	0.00

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

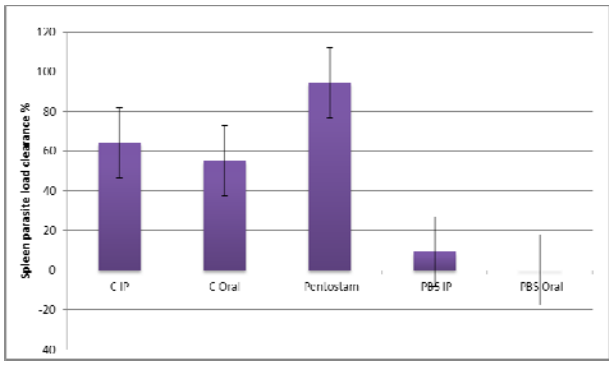

Fig 2: Showing spleen parasite load clearance after treatment

Discussion

The alarming increase in the development of drug resistant infections, emerging and reemerging infections have all contributed to intensive research for alternative therapy. Using plant based products are more advantageous as development of resistance by parasites is limited. Many plants have been documented to have antimicrobial activity. *A. secundiflora* leaf extracts has shown to contain a mixture of phenolic compounds, mainly anthrones (aloenin, aloenin B, isobarbaloin, barbaloin and other aloin derivatives [21]. On the other hand, *A. sativum* bulbs are known to contain organosulfur compounds present in the bulbs [13]. This chapter discusses the results regarding the aqueous crude extracts of combining *A. secundiflora* and *A. sativum* were tested against *L. major in vitro and in vivo.* The efficacy of combination therapy carried out in this study is presumed to the synergistic interaction of water soluble components of these plants. *Aloe secundiflora* and *A. sativum* water extracts showed low toxicity (IC_{50}) against healthy Vero cells of 467.09µg/ml and 540.14µg/ml respectively. The combination treatment of the extracts showed high toxicity of 391.79µg/ml which could suggest presence of synergistic effect since individual extracts had low toxicity. The combination had a lower toxicity than pentostam at 108.58µg/ml but the difference was not significant. The the cell viability of the combination therapy when closely compared with that of pentostam was 79.1% and 90.9% respectively. This showed that the combination therapy was less toxic as compared to the drug of choice, pentostam. This study reported a minimum inhibition concentration of 2000µg/ml and 5000µg/ml for *A. secundiflora* and *A. sativum.* The MIC for the single extracts was combined into several ratios and the 1:1 ratio supported the minimum growth of the *L. major* promastigotes. When more than one part of any single extracts was combined with one part of the other no life of *L. major* promastigote was observed.

Since the extracts did not stimulate the macrophages to produce sufficient amounts of nitric oxide, the actual mode of action by the extracts is not fully known. Presumably, the activity of *A. secundiflora* aqueous and methanolic extracts against *L. major* strains could be due to the ability of flavonoids to form complexes with the parasite cell wall and inhibiting the action of DNA polymerase [22].

In vivo studies indicated that there was a significant reduction of foot pad lesion sizes in *L. major* infected BALB/c mice that were treated intraperitoneally and orally with the aqueous extracts of *A. secundiflora* and *A. sativum* in a ratio of 1:1 (AF/AS), when compared to the mice treated with the controls PBS and pentostam. All the lesion size of all the mice decreased with time in the groups treated with the combination therapy either intraperitoneally or orally and pentostam but

increased in the negative control (PBS oral and IP). By the end of the fifth week of treatment, the lesions were not fully cleared. There was no significant difference in lesion size for the combination treatment of the oral and ip group. All of the infected mice treated orally or intraperitoneally with the combined aqueous extracts of *A. secundiflora* and *A. sativum* extracts had their lesion sizes reduce significantly when compared to those treated with PBS both orally and intraperitoneally. Amastigotes were present in the spleen after examination of smears suggesting that the combination therapy could not prevent visceralization. However, upon counting of the parasites in the experimental groups (combination treatment) and comparing them with those of controls there was a significant reduction which showed that the treatment with combination was effective

Conclusion

Aqueous extracts of *A. secundiflora, A. sativum* and combination did not stimulate production of significant amount of NO in peritoneal BALB/c macrophages, therefore, these extracts could have used other modes of action in killing the parasites hence if used together with other drugs the extracts could provide additive or synergistic effects in the control of different *leishmania* species. The aqueous extracts of *A. secundiflora, A. sativum* and their combination were relatively less toxic to Vero cells as compared to pentostam. Therefore, the combination extract was less toxic to Vero cells and BALB/c mice than the standard drug pentostam and due to this; they can provide better alternatives as drug for the control of leishmaniases. Intraperitoneally and orally administered combined aqueous of *A. secundiflora* and *A. sativum* extracts both reduced the lesion sizes of *L. major* infected BALB/c mice, but ip had a higher *in vivo* efficacy in reducing amastigotes burden in the spleens of *L. major* infected BALB/c mice with percentage parasite reduction of 64.13%, than oral with 55.48% respectively. Therefore, it can be concluded that the combination therapy using aqueous extracts of *A. secundiflora* and *A. sativum* at a ratio of 1:1, showed high *in vitro* anti promastigote activity, anti-amastigote, less toxic to Vero cells as compared to pentostam and the drug reduced lesions significantly but did not prevent visceralization.

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