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EFFICACY OF COMBINATION THERAPY USING EXTRACTS OF *ALOE SECUNDIFLORA* ENG L. AND *CALLISTEMON CITRINUS* WILLIAM C. IN *LEISHMANIA MAJOR* INFECTED BALB/C MICE
C. M. Ndeti, BSc, C. Kituyi, BAMLS, M. Ndirangu, BPMLS, Jomo Kenyatta University of Science and Technology, College of Health sciences, J. Ingonga, DMLT, Center for Biotechnology Research and Development (CBRD), KEMRI, M. L. Chimbevo, PhD, Medical Biochemistry Department, Mt Kenya University, J. O. Ochieng, BSc, MSN, College of Health sciences, Jomo Kenyatta University of Science and Technology, M. Barasa, BSc, MSc, Masinde Muriro University of Science and Technology, G. Kinuthia, PhD, Daystar University, E. M. Maina, PhD, University of Nairobi, V. C. S. Nyambati, PhD, College of Health sciences, Jomo Kenyatta University of Science and Technology and C. O. Anjili, PhD, Center for Biotechnology Research and Development (CBRD), KEMRI, P. O. BOX 54840-00100, Nairobi

Request for reprints to: Mr. C. M. Ndeti, College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology, P. O. Box 26086-00100, Nairobi, Kenya

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C. M. NDETI, C. KITUYI, M. NDIRANGU, J. INGONGA, M. L. CHIMBEVO, J. O. OCHIENG, M. BARASA, G. KINUTHIA, E. M. MAINA, V. C. S. NYAMBATI and C. O. ANJILI

ABSTRACT

***Background*: *Leishmania major* causes cutaneous leishmaniasis which leads to painful skin sores in humans. In the current study, efficacy of combination therapy of *A. secundiflora* and *C. citrinus* against *L. major* infected mice treated intra-peritoneally and orally was studied. Pentostam administered intra-peritoneally and phosphate buffered saline intra-peritoneally and orally were used as a controls.**

***Objective*: To determine the efficacy of combined therapy of *C. citrinus* and *A. secundiflora* extracts in *Leishmania major* infected BALB/c mice.**

***Design*: Experimental-Laboratory based study**

***Setting*: Kenya Medical Research Institute (*Leishmania* Department)**

***Subjects*: Eight weeks Male BALB/c Mice**

***Results*: The minimum inhibitory concentration (MICs) of aqueous extracts of *A. secundiflora* (A), and *C. citrinus* (B) were 2 mg/ml and 5 mg/ml respectively while the IC_{50} for the same extracts were 467.09μg/ml and 457.88μg/ml respectively. The combination of these extracts at ratio (1:1) supported minimal growth of *L. major* promastigotes and had IC_{50} of 58.45μg/ml as compared to MICs of 12.50μg/ml for Pentostam. The combination therapy had Infection rate (IR) of 19% and MI of 52.81% compared to Pentostam (IR=21% and MI=11.64%). The combination therapy reduced the footpad lesion size significantly ($P < 0.05$) just like the Pentostam control drug and no significant nitric oxide was stimulated. The oral and intra-peritoneal combination treatment reduced spleen amastigotes in mice by 73.46% and 78.12% corresponding to total LDUs of 10.87±0.64 and 8.96±0.82 respectively compared to Pentostam at 94.58% and LDU of 2.22±0.13. The difference between efficacy of Pentostam and that of combined extracts was almost significant ($t= 2.653$, $P= 0.057$).**

***Conclusion*: The combination therapy was active against *L. major* parasite, reduced lesion size significantly and did not prevent visceralisation but reduced spleen parasite load significantly.**

INTRODUCTION

The leishmaniases are caused by a protozoa parasite from over 20 *Leishmania* species of the genus *Leishmania* (Kinetoplastida: Trypanosomatidae) (1). They are endemic in areas of the tropics, subtropics, and southern Europe. Specifically, they are endemic in 88 countries (2). Currently, the leishmaniases have a wider geographical distribution pattern than understood before and are considered to be a major public health issue. An average of 350 million people are at risk of contracting leishmaniases and there are 1.3 million new cases and 20,000 to 30,000 deaths worldwide annually (3).

There are four main types of leishmaniases: In cutaneous forms, skin ulcers usually form on exposed

areas, such as the face, arms and legs. These usually heal within a few months; leaving scars (3). The main etiologic agents of cutaneous leishmaniases include *L. major*, *L. tropica*, *L. aethiopica, L. braziliensis*, *L. mexicana* and *L. panamensis*. Diffuse cutaneous leishmaniasis is characterized by disseminated nodules, an abundance of parasites throughout the course of the disease, the absence of parasite-specific cell-mediated immune response and a poor response to antimonials treatment (4). It may be caused by *L. amazonensis*, *L. mexicana* and *L. pifanol* and *L. aethiopica* (5). In mucocutaneous forms, the lesions can partially or totally destroy the mucous membranes of the nose, mouth and throat cavities and surrounding tissues (6). Muco cutaneous leishmaniases is caused by *L. aethiopica* and *L. braziliensis*. Cases due to *L. donovani*, *L. major* and *L. infantum* have been reported (7). Visceral leishmaniasis, also known as kalaazar, is characterised by high fever, substantial weight loss, swelling of the spleen and liver, and anemia. If left untreated, the disease can have a fatality rate as high as 100% within two years (8, 9). Kala azar is caused by *L. donovani* and *L.* (*infantum*) *chagasi* (10).

No cost effective control for leishmaniasis exists. Control of leishmaniasis is currently based on chemotherapy to alleviate the disease and on vector control to reduce transmission (11). Spraying of houses with insecticide is the most widely used intervention for controlling the sand fly vectors especially those that are endophilic whereas bed nets are ideal for sand flies that are endophagic (12). Other measures employed include use of traps to control the vector and use of repellents for personal protection. Studies have confirmed that natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans (13). Unfortunately, there is no protective vaccine available at the moment but Amphotericin B is used for those patients who do not respond to pentavalent antimony (14).

Aloe secundiflora is widespread, occurring in Sudan, Kenya and Tanzania with about 60 taxa recognized (15). The leaf components of *A. secundiflora* have been credited for antibacterial, antifungal and antiviral and anthelmintic medicinal properties. *Aloe secundiflora* have better active ingredients against *Lieshmania* parasites than methanolic extracts (16). Several studies done on *C. citrinus* have shown that the plant has therapeutic properties on several microbes. The water and methanolic extracts have antileishmanial effects against *L. major,* with water extracts showing better activity (17).

MATERIALS AND METHODS

Source of the plant extracts and extraction process: The leaves from *A. secundiflora* plant were collected from Ruai area, Nairobi, Kenya. The *C. citrinus* flowers were harvested from a selected area in Upper Hill, Nairobi. The plant materials were air-dried under a shade for 14 days and shred using a Gibbons electric mill. The ground material (200g) of each plant was soaked in 1000ml of distilled water and placed in a water bath at 80^{o}C for 1hour. The filtrate was freeze-dried, weighed and stored at -4^{0}C until required for use.

Experimental mice: Eight weeks old inbred male BALB/c mice were used for in vitro work (promastigote and amastigote assays) and for in *vivo* assays. The BALB/c mice were obtained from the stock maintained at the Kenya Medical Research Institute (KEMRI), Nairobi.

Cultivation of Leishmania major parasites and infection of mice: The *Leishmania major* (Strain IDUB/94=NLB-144) saline aspirate was taken from an infected mouse footpad and parasites were maintained as previously described (18). Briefly, the parasites were cultured in RPMI 1640 and liquid phase Schneider's *Drosophila* medium supplemented with 25% Fetal Bovine Serum (FBS), glutamine (2mM), Penicillin G (100U/ml) and streptomycin (100μg/ml) at 25^{0}C as previously described by Titus *et al.* (2010). The promastigotes were washed and purified 3 times in phosphate-buffered saline at 1500 rpm for 15 minutes. Stationary-phase metacyclic promastigotes of *L. major* (1x10^{6} cells/ml) were isolated from 5 to 7 day-old cultures. These parasites were used for *in vitro* and *in vivo* experiments. The left hind footpads of BALB/c mice were inoculated with 1 x 10^{6}stationary phase culture of *L. major* promastigotes in 40μl phosphate buffered saline (PBS) intra-dermally.

Preparation of the test drugs: Stock solutions of 1mg/ml concentration of crude extracts were made by weighing 10mg of single test extracts and dissolving 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 (Biological Safety Cabinet). The extracts were coded as A for *A. secundiflora*, B for *C. citrinus* and C for their combination. The combination drug (C) was made by weighing 2mg of *A. secundiflora* and 5mg of *C. citrinus* mix them and dissolved them in 1ml of PBS for *in vivo* work. The stock solutions were stored at -4^{o}C and retrieved when being used for *in vitro* and *in vivo*.

Cytotoxicity Assay: The assay was used to test the cytotoxicity of the individual extracts and combination therapy against Vero cells. The assay was carried out as previously described (19). Briefly, the African green monkey kidney (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^{0}C in a humidified 5% CO_2 atmosphere for 24 hours. The

Vero cells were harvested by trypsinisation, 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 microtitre plate at a concentration of 1×10^6 cells per ml of the culture medium per well and incubated at 37^{0}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 (A and B) and combination at ratio 1:1(200mg/ml:5000mg/ml) was added and serially diluted by a factor of three 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 controls wells comprised of Vero cells and medium while the blank wells had medium alone. 10μl 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 562 nm. Cell viability was automatically calculated at each concentration using the Mosmann (1983) formula. The IC_{50} values of the extracts were determined automatically using the Chemosen software program and the percentage cell viability calculated manually using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{Av. absorbance in duplicte. drug wels-av. blank wels}}{\text{Av.e absorbance in control wels}} \times 100$$

Evaluation of Minimum inhibitory Concentration (MIC): The MICs were determined as previously described (17). 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 (A and B) in concentrations that ranged between 5 mg/ml and 0.5 mg/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 AB was determined. The lowest concentration of the test plant extracts that supported the least survival of promastigotes growth was taken as the MIC and combination ratio that supported the least survival of promastigotes growth was noted.

Anti-Amastigote Assay: Three Balb/c mice were induced by injecting 2% starch intra peritoneally and left for 24 hours. The anti amastigote assay was carried out as previously described (20). Briefly, the mice were anaesthetized using 100µl pentabarbitone sodium (SagatalR). The peritoneal macrophages were obtained from mice upon disinfection of mice body surface with 70% ethanol. The abdominal skin was sheared dorso-ventrally to expose the peritoneum. Using a sterile syringe and needle, 10ml of sterile RPMI media was injected into the peritoneum. After shaking the mouse peritoneal macrophages were harvested by drawing the RPMI medium. The contents were transferred into a sterile 50ml centrifuge tube. The suspension was centrifugally washed at 2000 rpm (Hitachi, 05PR-22) for 10minutes and the pellet re-suspended in complete RPMI 1640 medium. Macrophages were adsorbed in 24-well plates and allowed to adhere for 4 hours at 37^{o}C in a 5% CO_2. Non adherent cells were washed with cold phosphate buffered saline (PBS) and the macrophages incubated overnight in RPMI medium. Adherent macrophages were infected with parasite: macrophage ratio of 6:1 and incubated at 37^{o}C in the 5% CO_2 for 4 hours. Unattached promastigotes were removed by extensive washing with PBS and the cultures incubated in RPMI for 24 hours. A onetime treatment of infected macrophages with the drugs was done. Pentostam was used as positive control drug for comparison of parasite inhibition. The medium and drug were replenished daily for 3 days. After 5 days, the monolayers of macrophages cells were washed with PBS at 37^{o}C, fixed in the methanol and stained with 10% Giemsa. The numbers of amastigotes were determined by counting at least 100 macrophages in duplicate cultures. The results were expressed in terms of infection rate (IR) and the multiplication index (MI) as used previously.

IR = Number of infected macrophages in 100 macrophages

$$\text{MI} = \frac{\text{No. of amast. in exp. cult./100 macrophages x 100}}{\text{No. of amast. in 100 cont. cult./100 macrophages}}$$

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 groups in different treatment groups were measured using a direct reading vernier caliper and lesion size calculations was done using the method as previously described (21) and expressed as follows:

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

Determination of spleen parasite loads in splenic impression smears: After 5 weeks of treatment, mice were sacrificed by inoculation with 60 mg/kg body weight of penta barbitone sodium. Spleen impression smear were made on clean microscopic slides. They were

left to dry for 15 minutes followed by fixation using absolute methanol. The method previously described (22) was used to quantify the parasite loads. Briefly, 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:

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

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

In vitro nitric oxide assay: Nitric Oxide (NO) release in macrophages culture was measured using the Greiss reaction for nitrites. Supernatants (100ul) were collected 48 hours after introducing the test drug into the culture medium. The assay was done in triplicate wells in the 96-well micro-titre plates. To achieve this, 60 ml of Greiss reagent A (1% Sulphonilmide in 1.2 M HCL) was added followed by 60ml of Greiss reagent B (0.35 N [1-naphthy] ethlenediamine). The plates were read at 540nm in the Enzyme Linked Immunosorbent Assay (ELISA) reader. Sodium nitrites in RPMI were used to construct a standard curve for each plate reading.

Statistical analysis: Data were analysed using Statistical package for social sciences (SPSS) software programme utilising 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 to carry out the study was obtained from Kenya Medical Research Institute (KEMRI) Ethical Review Committee.

RESULTS

Cytotoxicity test, cell viability and Minimum Inhibition Concentration (MIC) of the test extracts and control drug: When aqueous extracts were screened for cytotoxicity in Vero cells (P27), *A. secundiflora*, *C. citrinus* water extracts showed low toxicity (IC_{50}) against healthy Vero P27 cells of 467.09µg/ml, 457.88µg/ml respectively. The combination therapy showed high toxicity of 58.45µg/ml compared to the single plant extracts. However, the difference was not significant when compared using one sample test ($t = 2.481$, $P = 0.089$). The cell viability (%) increased as the drug concentration reduced showing that the drugs affected cell growth in an inverse manner. When promastigotes were exposed to varying concentrations of single plant extracts, combination of the extracts and the control pentostam drug, *A. secundiflora* showed MIC of 2000 µg/ml; *C. citrinus* of 5000 µg/ml, combination of *A. secundiflora and C. citrinus* at ratio of 1:1 and Pentostam of 12.5µg/ml.

Table 1

The IC_{50} (µg/ml) of the test extracts and their effects on Vero cells expressed as cell viability (%)

Drug	*A. secundiflora*	*C. citrinus*	*A. sec/C. cit (1:1)*	Pentostam
IC50 (µg/ml)	467.09	457.88	58.45	0.26
MIC (µg/ml)	2000	5000	1:1 (2000:5000)	12.5

Table 2

Vero cells viability (%) at various concentrations (µg/ml) of the experimental drugs

	Vero cells viability (%) at specific concentrations (µg/ml)							
Test extracts	1000.00	333.33	111.11	37.04	12.35	4.12	1.37	0.46
Control drugs								
A. secundiflora	10	68	112	110	111	113	106	100
C. citrinus	-70	79	57	93	118	108	112	100
A. sec/C. cit	76	40	10	79	85	93	100	100
Positive control	Vero cells viability (%) at specific concentrations (µg/ml)							
	100	50	25	12.5	6.25	3.125	1.563	
Pentostam	74.83	83.74	103.17	103.79	104.65	105.65	110.92	
Negative control* (MEM)	Average viability (%) of Vero cells ±S.D = 96.26± 0.46							

*The % viability in the negative control was the average for viabilities of Vero cells for MEM in microtitre plates A and B.

Anti-Amastigote assay of the test extracts: Different concentrations of aqueous single plant extracts of *A. secundiflora* (A), *C. citrinus* (B) and their combination (C) at a ratio of 1:1 were tested for their efficacy against amastigotes in macrophages. In order to determine the extent of infection, the number of infected macrophages and the number of amastigotes per 100 macrophages was determined. Infection rates were the number of parasites that actually infected the macrophages. One way ANOVA analysis of the IR% of A, B at 125µg/ml and C (1:1) closely compared to pentostam at concentration of 50µg/ml and RPMI showed no significant difference with $F_{(1,3)} = 2.242$ and P = 0.446. Comparison of IR% of the test drugs at 31.25µg/ml (57%, 51%) and combination, C, (19%) with the controls, pentostam at 12.5µg/ml (37%) and RPMI (71.5%) showed a less significant difference ($F_{(1,3)} = 21.348$, P = 0.157). The multiplication index of amastigotes of aqueous *C. citrinus* (B) compared to pentostam (P = 0.015) and pentostam compared to RPMI (P = 0.003) was significantly different. The MI% of *C. citrinus* and the combination drug (C), was significantly different with a P= 0.032. The combination (C), and test drug A's (*A. secundiflora*) MI% closely compared to that of pentostam was not significantly different P = 0.602 and 0.225 respectively.

Effects of combination therapy (C) on lesion sizes: Combined extracts of *A. secundiflora* and *C. citrinus* (C) administered intra-peritoneally and orally to infected Balb/c mice, the foot pad lesion sizes decreased. 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 =9.127 and P = 0.001. *Post hoc* tests of multiple comparisons (Games-Howell) showed a significant difference between combination (C) oral against PBS ip (P = 0.042), combination (C) oral against PBS oral (P = 0.044). Pentostam against PBS ip and PBS oral were also significantly different with P values of 0.042 and 0.047 respectively.

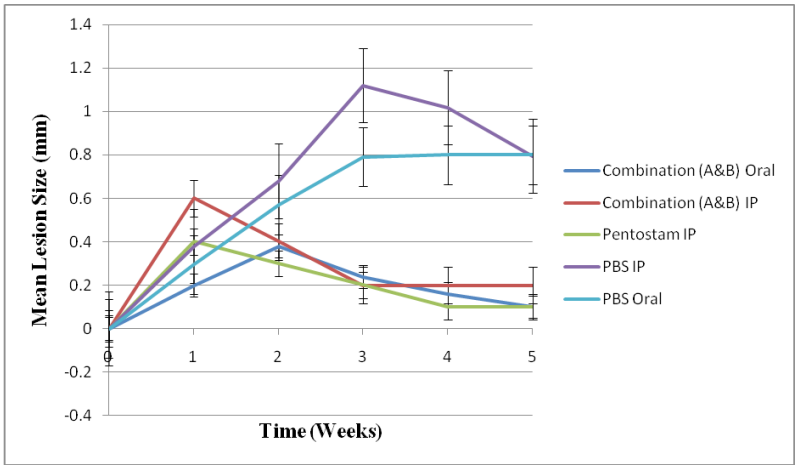
Table 3

Showing average lesion size (mm) and Post hoc (Games-Howell) test P values againstPBS oral in BALB/c mice infected with L. major treated with combination aqueous test extracts and controls administered orally or intra-peritoneal (ip) over 5 weeks (weeks) period

		Mean footpad swelling (mm) a/Mean lesion size					
Drugs	Route	Wk1	Wk2	Wk3	Wk4	Wk5	Drug vs PBS oral P-value
A.sec/C.cit	IP	0.60	0.40	0.20	0.20	0.20	0.792
A.sec/C.cit	Oral	0.20	0.38	0.24	0.16	0.10	0.044*
Pentostam	IP	0.40	0.30	0.20	0.10	0.10	0.047*
PBS	Oral	0.29	0.57	0.79	0.79	0.79	N/A
PBS	IP	0.38	0.68	1.12	1.02	0.79	0.878

Figure 1

The foot pad swelling after treatment with the experimental drugs for five weeks.Data represent mean lesion size ±S.E, (n=5), five weeks after treatment



Estimation of number of Leishmania parasites in the infected BALB/c mice splenocytes: The spleen index (%), Leishman-Donovan Unit (LDU), total Leishman-Donovani Unit (total LDU) and percentage parasite reduction in spleens of the infected Balb/c were determined to show the efficacy of the drugs. One way ANOVA comparison of total LDU showed there was significant difference of the combination therapy and the controls (F (4, 12) =113, P = 0.001). The PBS oral treatment was assigned arbitrary assigned 100% parasite load and used as standard to calculate the percentage (%) parasite reduction of the other drugs. The efficacy of the combination in reducing parasite load in the spleens of *L. major* infected Balb/c mice was the highest at 78.12% as compared to oral route at 73.46%. The positive control (pentostam) had the highest parasite reduction percentage of 94.58% as expected but some reduction of 9.28% was observed in negative control PBS ip treatment. A paired t-test comparison of the parasite clearance showed a near significance level (t= 2.653, P= 0.057).

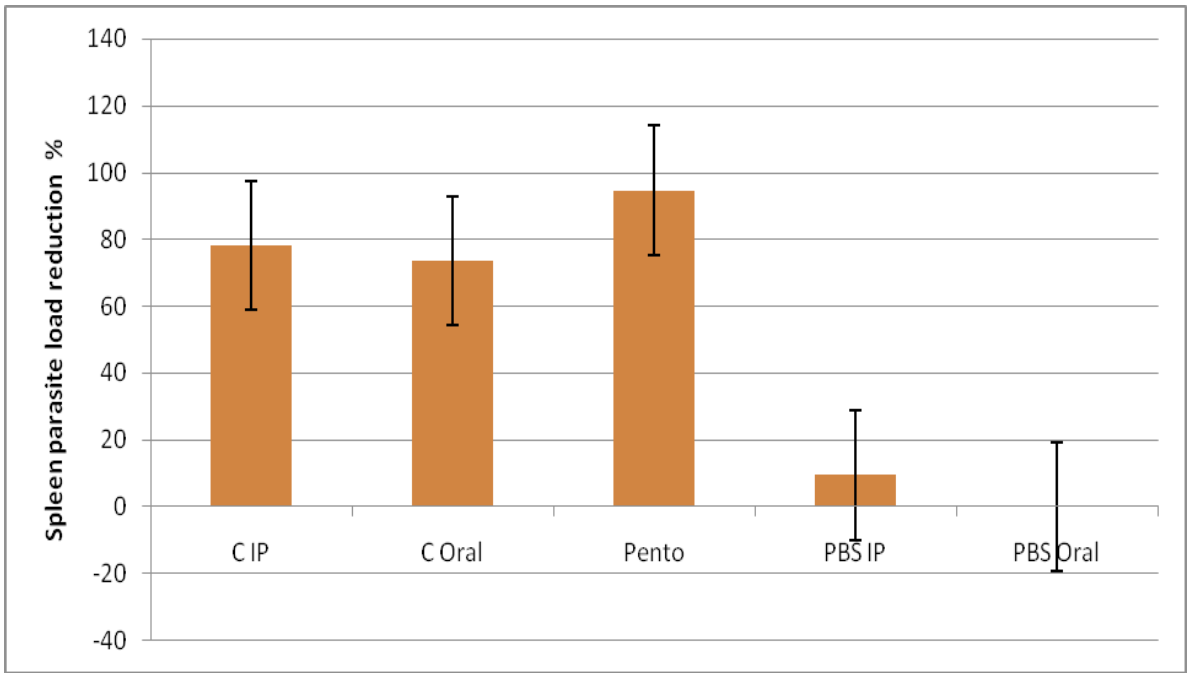
Table 4

Shows the average spleen weight, index, LDU and total LDU for groups of L. major infected BALB/c mice that were treated with combined test aqueous and controls

Combination and Control	Route	Ave spleen weight ±SE	Ave spleen Index (%) ±SE	Ave LDU ±SE	Ave total LDU ±SE(× 1000)	% Parasite reductiona
A. sec/C.citri	IP	0.13± 0.02	0.67 ± 0.42	0.35± 0.02	8.96 ± 0.82	78.12
A. sec/C.citri	Oral	0.13± 0.01	0.53 ± 0.45	0.42± 0.02	10.87±0.64	73.46
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

Figure 2

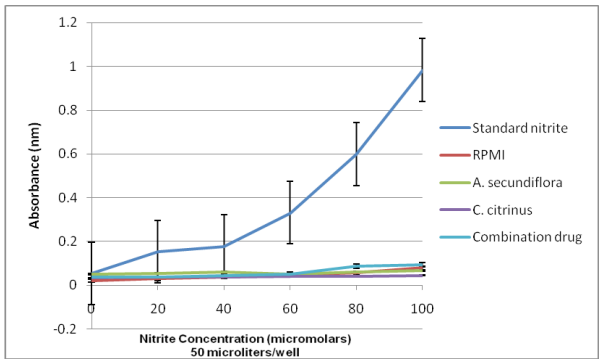
Spleen parasite load reduction/clearance % after treatment with the experimental drugs. Data represent parasite load reduction/clearance % ±S.E, (n=5), five weeks post treatment



Stimulation of Nitric Oxide production activity the test drugs: The nitric oxide production in supernatants of macrophage culture treated with water plant extracts of *A. secundiflora*, *C. citrinus* and their combination were determined using a representative standard curve for samples at concentrations between 0 and 1000µg/ml. No significant nitric oxide levels ($P > 0.05$) were produced compared to the negative control. None of the samples had Optical Density (OD) readings of more than 0.10 indicating that less than 2µm of nitric Oxide was produced compared to negative controls that produced similar levels.

Figure 3

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)



DISCUSSION

Various experimental studies have shown that several plants in different genera contain compounds that have anti-leishmanial activity and can be used as alternative therapies although their effectiveness differs according to the active compounds present in the individual plants (23). After successful *in vitro* assays, *in vivo* assay was conducted to measure the efficacy of the combination of *A. secundiflora* and *C. citrinus* by measuring its effect in Balb/c mice with well-developed lesions. Combination therapy of *A. secundiflora* and *C. citrinus* water extracts showed low toxicity against Vero cells hence justifying that these plants are safe for herbal medicine. The combination therapy inhibited the infection of macrophages more effectively than the single extracts suggesting synergistic effects of the compounds in both the plants. Production of nitric oxide (NO) by infected macrophages has been reported to be one of the mechanisms that enhance the killing of *Leishmania* parasites in the phagocyte (24). The combination of the *Aloe secundiflora* and *C. citrinus* also did not produce significant levels of nitric oxide and it means that other mechanisms to kill the parasites in the macrophages were used probably such as forming complexes with the parasite cell wall affecting cell-linked processes there by inhibiting its growth, inhibiting the action of the DNA polymerase, inhibiting cell enzyme activities like inhibiting the action of the DNA polymerase among other modes of action.

There was a significant reduction of foot pad lesion sizes in *L. major* infected Balb/c mice that were treated through intra-peritoneally and orally with blend of aqueous extracts of *C. citrinus* and *A. secundiflora* in a ratio of 1:1 (AB), when compared to the mice treated with the controls PBS (ip and oral) and pentostam. All the lesion size of all the mice decreased in combination therapy either intra-peritoneally or orally and pentostam but increased in the negative control (PBS oral and IP). However, the lesions did not fully clear by the end of fifth week of treatment. *L. major* parasites are known to invade the liver and spleen (visceral organs), a process known as visceralisation. The *L. major* parasites were able to invade these organs but the combination therapy significantly cleared them significantly as depicted by the percentage parasite reduction that ranged from 73.46 to 75.12%. The efficacy of the combined extracts at a ratio of 1:1 might have been influenced by the experimental dosage used.

A mixture of phenolic compounds, mainly anthrones (aloenin, aloenin B, isobarbaloin, barbaloin) and other aloin derivatives from *A. secundiflora* have been determined from the leaf exudate (25). Various types of secondary metabolites have been isolated from *Callistemon* family, including triterpenoids (26), Phloroglucinol derivatives (27), C-methyl flavonoids (28) and tannins (29).The efficacy of the combination therapy reported in this study is presumed to be attributed to the synergistic interaction of water soluble compounds of these plants.

In conclusion, the combination therapy of *A. secundiflora* and *C. citrinus* water extracts produced inhibitory activities against *L. major* promastigotes and amastigotes. It marked higher antileishmanial activities though not of comparative concentrations than of the pentostam. The results also showed that the combination therapy had slightly higher toxicity against Vero cells as compared to the standard drug pentostam. The combination therapy did not stimulate the macrophages to produce significant amounts of nitric oxide; hence it could be acting directly on the parasite rather than stimulating nitric oxide production to kill the parasites. Further laboratory testing and clinical use of this combination in other models of leishmaniasis for example using non-human primates such as vervet monkeys could provide strong evidence that the combination of *A. secundiflora* and *C. citrinus* could be used as possible drug for leishmaniasis.

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