

Research Articles

Combination and monotherapy of *Leishmania major* infection in BALB/c mice using plant extracts and herbicides

Judith A. Makwali¹, Frederick M.E. Wanjala¹, Josyline C. Kaburi², Johnstone Ingonga², Wabwoba W. Byrum³ & Christopher O. Anjili²

¹School of Science, Moi University, Eldoret; ²Center for Biotechnology Research and Development (CBRD), Kenya Medical Research Institute (KEMRI), Nairobi; ³Mombasa Technical Training Institute, Department of Applied Sciences, Mombasa, Kenya

ABSTRACT

Background & objectives: Leishmaniasis is a growing health problem in many parts of the world. Efforts to find new chemotherapeutics for leishmaniasis remain a priority. This study was carried out to determine the effect of combination and monotherapies using plant extracts and herbicides on *Leishmania major* infection in BALB/c mice.

Methods: The herbicides and saponin extract were purchased from Sigma. Roots of *Plumbago capensis* were collected from Karura forest, Nairobi, Kenya. Plant extractions were done in KEMRI at Center for Traditional Medicines and Drugs Research.

Results: Lesion sizes after infection of BALB/c mice were similar in all the experimental groups till the onset of therapeutic treatments ($p > 0.05$). At 15 days post-treatment, significant differences ($p < 0.05$) were discerned in the lesion sizes of the BALB/c mice in all the mono- and combined-treated groups. However, the combined therapies caused total elimination of the parasites from the lesions and significantly reduced parasite burden in liver and spleen compared to the untreated controls at the end of the experiment.

Interpretation & conclusion: The results of this study demonstrate that combination therapy using alternative administration of saponin, acriflavine, trifluralin and plumbagin is effective in treating *L. major* infection in mice. In this regard, an investigation into the efficacy of these combined therapies against other *Leishmania* strains should be explored further. Furthermore, studies with these combination therapies should be done on non-human primates such as the vervet monkey (*Cercopithecus aethiops*).

Key words Antileishmanial activity; herbicides; *Leishmania major*; plant extracts

INTRODUCTION

Leishmaniasis is a zoonotic disease of major public health and veterinary importance, affecting 88 countries with at least two million new cases each year and 367 million people at risk¹. It is caused by the protozoan parasite of the genus *Leishmania* and transmitted to the mammalian host by the bite of the phlebotomine sandfly. Complicated clinical symptoms range from the skin lesions of cutaneous leishmaniasis (CL) to the fatal visceral leishmaniasis (VL), making the disease an important health problem in the world. *Leishmania* parasite has a complex life cycle that includes two different developmental forms. The parasites replicate as promastigotes and develop into infective metacyclic promastigotes in the sandfly vector, and the infective promastigotes invade the macrophages and differentiate into amastigotes in the mammalian host².

Since vaccines against leishmaniasis are not available,

the control of this disease depends on prompt diagnosis and chemotherapy on infected humans and dogs. Chemotherapy of this disease has relied mainly on pentavalent antimonials, amphotericin B (AMB), and pentamidine³. These drugs are toxic and difficult to administer because of their long-term treatment and high cost^{4,5}. Though targeting of antileishmanial agents in liposomes, microspheres, and nanoparticles can successfully increase the efficacy and reduce toxicity of antileishmanial drugs, the limitations of these delivery strategies include non-specific accumulation of reticuloendothelial system (RES), instability of the liposomal formulations, and the incomplete degradation of polymeric delivery systems⁶. Therefore, investigations are still in progress to discover new antileishmanial drugs.

Mitotic disrupter herbicides like trifluralin are good lead compounds for antiparasitic drugs because these have already been shown to lack activity against mammalian

cells and are inexpensive to produce. Trifluralin acts directly with the major microtubule protein, tubulin, leading to disruption of mitosis. *Leishmania* species also appear to have the same biochemical mechanism of inhibition as plants, since trifluralin was found to specifically inhibit polymerization of *Leishmania* tubulin, but not mouse tubulin, *in vitro*⁷. Thus, leishmanial tubulin appears to be more similar to plant tubulin than to mammalian tubulin, with distinct structures that can be targeted by drugs. Although, the antimicrobial dinitroaniline herbicides show great potential as antiprotozoal compounds, disputed indications of potential carcinogenicity will probably keep trifluralin from being developed for human use. However, in 1993 Chan and his co-workers indicated that an impurity contaminant in trifluralin, not trifluralin itself, may be responsible for the observed *in vitro* activity against *Leishmania* species⁸. The active impurity in trifluralin is chloralinal, an intermediate in the synthesis of trifluralin. However, there is no commercial impetus to reformulate this drug.

On the basis of ultrastructural morphology and sequence comparison of small subunit ribosomal RNAs, *Leishmania* and *Trypanosoma* are phylogenetically related and both species have a kinetoplast that contains superhelical DNA⁹. As early as 1968, Simpson¹⁰ showed that acriflavine in low concentrations, through selective inhibition of replication of kinetoplast DNA and not nuclear DNA is able to induce dyskinetoplasia in cultures of *L. tarentolae*. During growth in acriflavine cultures, cells were seen to exhibit an increasing impairment of colony-forming ability and rate of respiration. It was suggested that the mechanism of action could be photo-oxidization of the kinetoplast DNA. It is, therefore, possible that acridine compounds, either modified or used in combination with other drugs can be used to treat the leishmaniasis.

Saponins on the other hand are high molecular weight glycosylated plant secondary metabolites, consisting of a sugar moiety linked to a triterpene or steroid aglycone that show various kinds of biological activity and have been used widely as anti-inflammatory, antitumor, anti-HIV, and antifungal agents¹¹. Consequently, triterpenoid saponin structures have become the synthetic targets of many research groups¹². Detergent properties are the typical characteristics of saponins. These produce stable foam when dissolved in water, which is why some saponin-containing plants have been used as soaps for hundreds of years. Saponin containing plants are used as traditional medicines, especially in Asia, and are intensively used in food, veterinary and medical industries¹³. Antileishmanial activity has been reported for *Hedera*, *Dracaena* and *Yucca* saponins¹⁴, but their value as drug candidates has not been

fully assessed. Saponins inhibit the growth of the promastigote forms by acting on the membrane of the parasite where they induce a drop in membrane potential¹⁵.

Plumbagin (2-methoxy-5-hydroxy-1,4-naphthoquinone) is a natural naphthoquinone possessing various pharmacological activities such as antimalarial¹⁶, antimicrobial¹⁷, anticancer¹⁸, cardiotoxic¹⁹ and antifertility action²⁰. At present, the most exploited source of plumbagin is the roots of *Plumbago* spp. (*Plumbago europea*, *P. rosea*, *P. zeylanica* and *P. capensis*). However, these plants grow quite slowly and it takes long before the roots are suitable for use²¹. In this study, the roots of *P. capensis* were used for plumbagin extraction. Therefore, there is need to search for alternative sources of plumbagin that grow quickly.

Advances in antileishmanial chemotherapy including development of new drugs have made combination chemotherapy a real possibility. Drug combinations aim to delay or prevent the emergence of resistance, shorten the course of treatment and lower required doses. Other potential advances include convenience, better compliance and lower costs²². In this study, trifluralin, acriflavine, saponin and plumbagin were used in alternate combination of two drugs against *L. major* infection BALB/c mice.

MATERIAL & METHODS

Leishmania parasites

Metacyclic promastigotes of *L. major* (IDUB/KE/83=NLB-144 strain) were used. This strain has been maintained by cryopreservation and *in vitro* culture, and periodic passage in BALB/c mice at Kenya Medical Research Institute (KEMRI). Parasites were grown at 26°C in Schneider's *Drosophila* medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS).

Mice

Female BALB/c mice ranging from 8-12 wk old were used in this study. They were strictly maintained under the rules and regulations of the Animal Care and Use Committee-KEMRI (ACUC-KEMRI, Kenya) in specific pathogen-free conditions with laminar air flow. All mice were inoculated intramuscularly with 2×10^6 stationary phase promastigotes of *L. major* on the left hind footpad (LHFD). All animal experiments were approved by the Kenya Medical Research Institute (KEMRI) Committee. Lesion sizes were estimated weekly for 2.5 months post-infection. Infected control mice were not treated. In order to compare the effect of the drug, the difference between the infected left hind footpad and the right hind footpad was taken on a weekly basis according to the method of Nolan and

Farrell²³. Briefly, the lesion size was measured in two dimensions (D and d) at right angles to each other with a caliper gauge, and the lesion size(s) determined by the following formula: $S = (D \times d)/2$. The mice were anesthetized with sodium thiopental (50 µg/g of body weight) and sacrificed at the end of the experiment and their spleen and liver were removed for parasite load determination. This was done because *L. major* infection in BALB/c visceralises.

Drug administration

After the skin lesion was confirmed (two months after the inoculation), the infected mice were randomly assigned to seven groups of 10 mice each. The drugs were subcutaneously injected into the mice twice daily for seven days in different schemes of drug combinations with a drug concentration equivalent to the $IC_{50}/24$ h of each of the six drugs used. In some cases the treatment was withheld up to four months to evaluate the efficacy of the treatment in advanced infection. For the experimental handling, the groups had been divided as follows: Group 1 received a dose each of acriflavine and saponin; Group 2 received a dose each of trifluralin and acriflavine; Group 3 received a dose each of trifluralin and plumbagin; Group 4 received a dose each of saponin and plumbagin; Group 5 received a dose each of trifluralin and saponin; and Group 6 received a dose each of acriflavine and plumbagin. The control group was left untreated. Similar groupings were set up for monotherapy treatments.

Drugs and plant extracts

Trifluralin, acriflavine and saponin extract were purchased from Sigma. Roots of *P. capensis* were collected from Karura forest, Nairobi, Kenya. Plant extractions were done in KEMRI at Center for Traditional Medicines and Drugs Research. Reference drugs included sodium stibogluconate (Pentostam; Sigma), pentamidine isethionate (Sigma), and amphotericin B (AMB) (Fungizone; Squib).

Extract preparation

Roots of *P. capensis* were oven dried at 40°C and powdered (5 mm of particle dia). The plant (9.5 g) was extracted with 300 ml of chloroform in the Soxhlet apparatus for 5 h to obtain high percentage of plumbagin. All the solutions were evaporated to dryness under reduced pressure. The extraction efficiency was defined as follows:

$$\text{Mass of extraction} = \frac{\text{Mass of extracts}}{\text{Mass of dried material roots}} \times 100$$

Compounds used in this study

Plant extract solutions for biological testing and the reference drugs were prepared in 100% dimethyl sulfoxide (DMSO) at 20 mg/ml. Reference drugs included sodium stibogluconate (Pentostam; Sigma), amphotericin B (Fungizone; Squib), pentamidine isethionate (Sigma), trifluralin (Sigma) and acriflavine (Sigma). All the prepared drugs were stored at 4°C and retrieved only during use. In most experiments, pentostam was used as a reference drug.

Determination of parasite burdens

The assessment of the amastigote burden was carried out by blinded microscopic enumeration with Giemsa-stained liver and spleen touch prints. The total amastigote burdens were calculated as Leishman Donovan units (LDU, number of amastigotes per 1000 nucleated cells \times organ weight [in grams] $\times 2 \times 10^5$), according to Stauber's formula²⁴. The percent efficacy was calculated as $[1 - (\text{mean amastigote load in treated mice} / \text{mean amastigote load in NTC})] \times 100$. An LDU reduction of at least 80% was adopted as the minimal criterion for drug efficacy. Collection of target organs and determination of amastigote burdens were not done for mice that succumbed due to severe clinical disease.

Statistical analysis

All the experiments were carried out thrice. The mean and standard deviation of at least three experiments were determined. Statistical analysis of the differences between mean values obtained for the experimental groups was done by analysis of variance (ANOVA) and Student's *t*-test. *P*-values of 0.05 or less were considered significant.

RESULTS

Effect of monotherapy on *L. major* lesion development

BALB/c mice infected on the LHFD with *L. major* promastigotes showed skin lesions as a single nodule with or without ulceration two months after inoculation. The lesions developed to full size in most of the infected mice. Efficacy of monotherapy treatments on the development of lesion sizes in BALB/c mice is presented in Fig. 1. Lesion sizes after infection of the BALB/c mice were similar in all the treatment groups till the onset of therapeutic treatments. Treatment was started after two months of infection. At 15 days post-treatment, significant differences were discerned in the lesion sizes of the BALB/c mice. BALB/c mice-treated using acriflavine and trifluralin produced significantly larger lesion sizes than those being treated using saponin and plumbagin. However, after one month of monotherapy treatment, lesion sizes in all the BALB/c mice

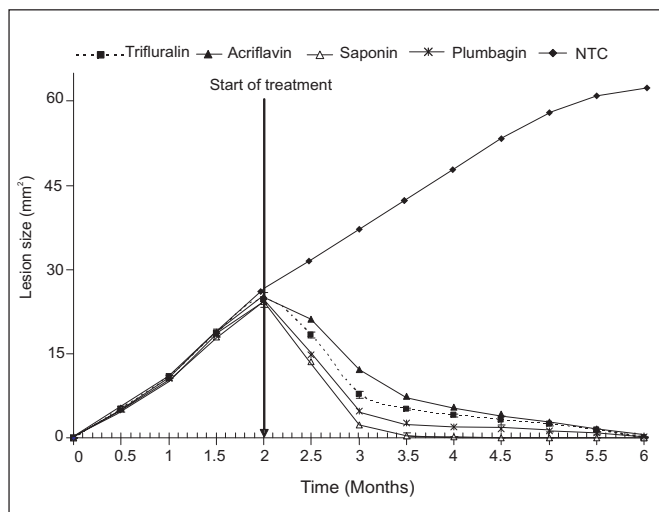


Fig. 1: Lesion sizes of mice during infection, pre- and post-monotherapeutic treatments.

had differentiated ($p < 0.05$) with saponin maintaining significantly lower lesion size in the BALB/c mice. Lesions

were completely eliminated after monotherapeutic treatment using saponin after two months post-treatment while all the other monotherapy treatments reduced the lesions after four months post-treatment.

Effects of monotherapy on liver and spleen parasite burdens

A single 0.2 mg/kg dose of saponin, plumbagin, trifluralin and acriflavine resulted in 97.8, 98.8, 86.4 and 85.4% reductions in liver amastigote burden on Day 7 post-treatment, respectively, whereas on Day 56 post-treatment the liver amastigote burden in mice-treated with saponin, plumbagin, trifluralin and acriflavine were 92.06, 92.44, 82.42 and 80.44%, respectively. The severity of infection in the non-treated controls (NTC) increased dramatically between Day 7 and Day 56, as illustrated by a 4-fold increase in the liver amastigote burden (from 149.3×10^6 LDU on Day 7 to 597.2×10^6 LDU on Day 56) (Table 1). Despite the apparently adequate parasite control by

Table 1. Effects of monotherapy treatment on *L. major* infection in BALB/c mice

Treatment group ^a	Body wt (g)	Days post-infection	Relative weight (%) [†]		LDU (10^6) (% efficacy) [‡]	
			Liver	Spleen	Liver	Spleen
Plumbagin	25.8 ± 0.7	8	4.3 ± 0.29	0.46 ± 0.07	11.72 ± 0.2 (98.8)	0.53 ± 0.08 (97.6)
Saponin	24.5 ± 0.5	8	4.2 ± 0.31	0.43 ± 0.06	13.23 ± 0.3 (97.8)	0.52 ± 0.07 (97.6)
Trifluralin	25.9 ± 0.8	8	5.2 ± 0.17	0.58 ± 0.1	20.25 ± 0.44 (86.4)	0.63 ± 0.5 (84.1)
Acriflavin	25.5 ± 0.5	8	5.6 ± 0.12	0.59 ± 0.25	22.06 ± 0.35 (85.4)	0.69 ± 0.3 (83.3)
Pentostam (250 mg/kg)	26 ± 2.8	8	4.4 ± 0.24	0.45 ± 0.23	11.98 ± 0.52 (98.6)	0.56 ± 0.11(95.6)
NTC	24.5 ± 1.4	8	6.75 ± 2.19	0.69 ± 0.08	149.3 ± 12.84	3.18 ± 0.25
Treatment group ^b						
Plumbagin	17.4 ± 0.7	35	6.45 ± 0.55	0.69 ± 0.09	5.63 ± 0.48 (99.6)	0.51 ± 0.08 (97.4)
Saponin	18.1 ± 0.7	35	5.69 ± 0.59	0.58 ± 0.08	3.23 ± 0.28 (99.9)	0.56 ± 0.07 (97.6)
Trifluralin	16 ± 0.8	35	9.60 ± 0.55	1.10 ± 0.18	102.59 ± 0.45 (93.1)	0.48 ± 0.54 (84.1)
Acriflavin	15.5 ± 0.5	35	9.26 ± 0.31	1.18 ± 0.05	13.66 ± 0.30 (90.8)	0.37 ± 0.14 (78.6)
Pentostam (250 mg/kg)	17.2 ± 0.5	35	4.52 ± 0.22	0.45 ± 0.22	0 ± 0 (100)	
NTC	17.5 ± 0.6	35	7.01 ± 0.4	1.3 ± 0.3	746.5 ± 80.54	
Plumbagin	17 ± 0.56	56	5.17 ± 0.9	0.39 ± 0.12	11.28 ± 1.3 (92.44)	1.51 ± 0.14 (83.9)
Saponin	18.3 ± 1.54	56	5.32 ± 0.63	0.52 ± 1.4	27.24 ± 2 (92.06)	1.48 ± 0.21 (86.8)
Trifluralin	16 ± 1.67	56	5.3 ± 0.85	0.32 ± 0.05	29.47 ± 3.1(82.40)	2.73 ± 0.11 (77.0)
Acriflavin	15 ± 2.09	56	4.18 ± 0.30	0.39 ± 0.08	38.4 ± 4.25 (80.44)	3.06 ± 0.18 (66.7)
Pentostam (250 mg/kg)	17 ± 0.57	56	6.25 ± 1.68	0.82 ± 0.88	11.85 ± 3.65 (92.1)	2.84 ± 0.68 (73.6)
NTC	17.5 ± 1.2	56	8.03 ± 0.91	2.01 ± 0.41	597.2 ± 75.7	12.51 ± 6.01
Plumbagin	14 ± 0.98	—	ND			
Saponin	15 ± 0.78	63	ND			
Trifluralin	14 ± 0.65	63	ND			
Acriflavin	15 ± 0.74	63	ND			
Pentostam (250 mg/kg)	15 ± 0.54	84	6.31 ± 0.92	1.25 ± 0.58	ND	ND
NTC	13 ± 0.75	84	—			

^aTreatment of acute infection on Day 1 post-infection at 0.2 mg/kg; ^bTreatment of chronic infection on Day 28 post-infection at 1.25 mg/kg; [†]Organ weight/body weight; [‡] $[1 - (\text{mean amastigote load in treated mice} / \text{mean amastigote load in NTC})] \times 100$; NTC — Non-treated control; LDU—Leishman Donovan units; ND — Not done.

the test compounds at 0.2 mg/kg (80.44–92.44% reduction in liver amastigote burden), deaths did occur and the survivors showed considerable weight loss (52%). Treatment with the test compounds at 1.25 mg/kg resulted in a high reduction in liver amastigote burden. However, one animal died on Day 46 and the overall body weight gain was low (5–7 g). By Day 35, control mice already showed liver infection (746.5×10^6 LDU), poor health status, and a body weight loss of about 25%. Control of liver infection was obtained with saponin at 0.8 mg/kg and pentostam at 250 mg/kg/body weight, with 99.9 and 99.4% reductions within 7 days of treatment, respectively; and control of liver infection was obtained with plumbagin, trifluralin and acriflavine at 1.25 mg/kg with 99.6, 93.1 and 90.8% reductions within 7 days of treatment, respectively. However, a net body weight loss was noted in all the animals, and the spleen remained positive for amastigotes.

Effect of combination therapy on L. major lesion development

In the initial testing for the antileishmanial activity of independent drugs the order of potency was as follows in decreasing strength: saponin (Sap) > Plumbagin (Plgn) > Trifluralin (Tri) > Acriflavine (Acri). After 7 days of treatment, twice daily with saponin, the parasites in all the infected animals disappeared from the lesion area after 14 accumulated drugging, and the open lesions completely healed in a period of 30 days (Fig. 1). In the independent drug therapy (monotherapy), the parasites reappeared in the skin at the site of infection and in the surrounding skin area 90 to 150 days after the end of the treatment. It was a result of a successful combination of acriflavine and saponin that prompted the following series of experiments. Each drug combination was examined. In this case the lesions healed, with only the untreated mice dying.

Loss of weight and discoloration was apparent in untreated mice and mice treated with the drugs of choice namely AMB and pentostam. The treated mice, on the other hand, increased in weight. Of the six drugs of choice tested against the disease, sodium stibogluconate and pentamidine were effective but some mice treated with these drugs of choice died as a result of toxicity. None of the mice treated with combination therapy died. No formulation was toxic to the experimental mice. Combination treatment comprising of alternate administration of test compounds caused total elimination of the parasites from the lesion at the end of the treatment (Fig. 2). After 7 days of treatment, twice daily, with these combinations, the parasites in all the infected animals disappeared from the lesion area, and the open lesion completely healed in a period of 30 days.

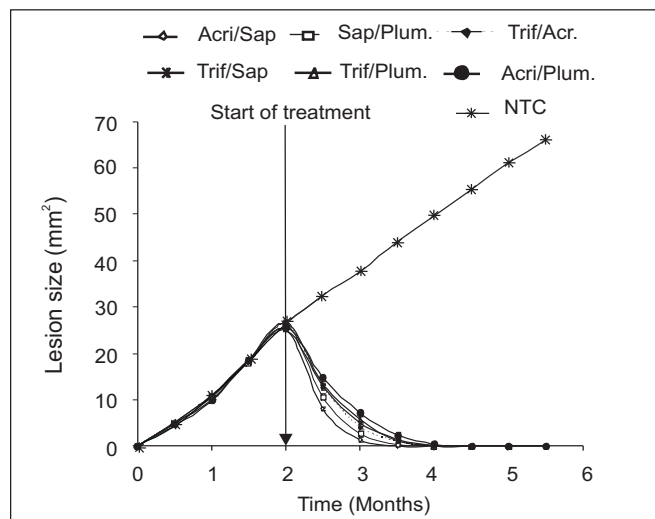


Fig. 2: Lesion sizes of mice during infection, pre- and post-combination therapy treatments.

Efficacy of combined treatments on the development of lesion sizes in BALB/c mice is presented in Fig. 2. Lesion sizes after infection of the BALB/c were similar in all the treatment groups till the onset of therapeutic treatments ($p > 0.05$). Treatment was started after two months of infection. At 15 days post-treatment, significant differences ($p < 0.05$) were discerned in the lesion sizes of the BALB/c mice. BALB/c mice being treated using acriflavine/plumbagin produced significantly larger lesion sizes than those being treated using acriflavine/saponin. However, after one month of monotherapy treatment, lesion sizes in all the BALB/c mice had differentiated ($p < 0.05$) with acriflavine/saponin maintaining significantly lower lesion size in the BALB/c mice. Lesions were completely eliminated after combination treatment using acriflavine/saponin after one and half months post-treatment, while all the rest of the combinations cleared the lesions after two months post-treatments, except for acriflavine/plumbagin treatment that reduced lesions after four months post-treatment.

Effects of combined treatments on the amastigote liver and spleen burdens

Results of therapeutic treatments on the liver and spleen LDU are presented in Fig. 3. For both the liver and spleen, the non-treated controls (NTC) had the highest LDU whereas there was a decline in the LDU of the liver from Day 10 to 52, the LDU increased during the same time period. After 10, 30 and 52 days of treatments, significantly ($p < 0.05$) higher LDU in the liver and spleen occurred in acriflavine/plumbagin combination followed by trifluralin/plumbagin and the lowest LDU occurred in the trifluralin/acriflavine combination therapies.

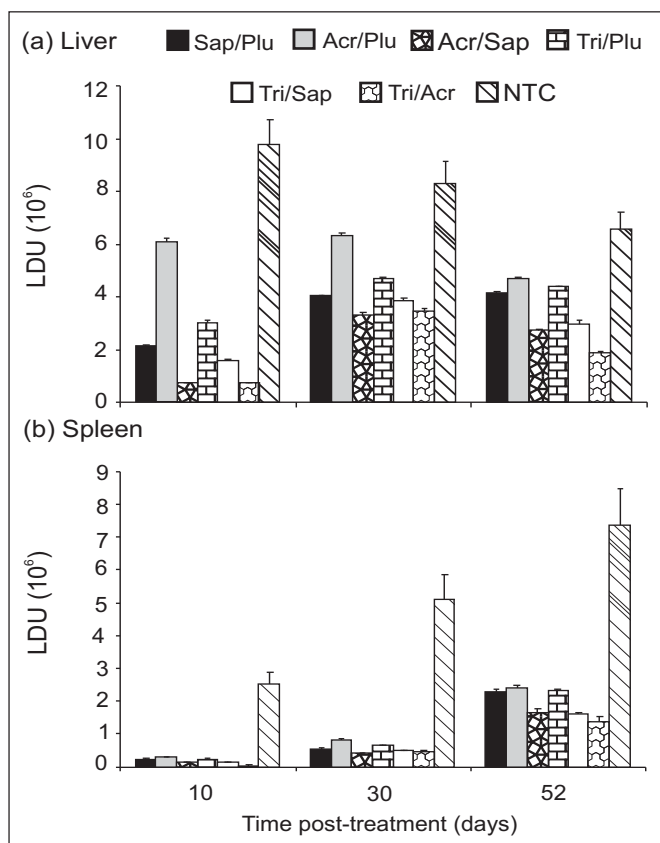


Fig. 3: Liver and spleen LDU on Day 10, 30 and 52 post-treatment.

DISCUSSION

Leishmania major, the aetiological agent of CL on humans, is a parasite of the skin. However, in the BALB/c mice, *L. major* produces visceral infection in addition to the local lesion at the point of inoculation. Thus, it was a suitable model for this study. The fact that a spontaneous healing cannot be achieved in these mice indicates that the complete healing of the lesions is due to the combination therapy administered only. Therefore, to better evaluate if the drugs effectively reduced the parasites burden in the liver and spleen after treatment, short-term and long-term efficacies for the mono and combination therapies were determined. For both the liver and spleen, the non-treated controls had the highest LDU. Significantly ($p < 0.05$) higher parasite load in the liver and spleen occurred in acriflavine/plumbagin combination followed by trifluralin/plumbagin and the lowest LDU occurred in the trifluralin and acriflavine combination therapies.

These results indicate that acridine and dinitroaniline herbicides with the plant extracts are promising lead compounds for development as novel antileishmanial agents. DMSO was used in all the drug formulations because it has been reported to increase drug penetration²⁵. Com-

bined therapy of saponin and acriflavine was the most effective in reducing both the lesion sizes and hepatic parasite load; followed by saponin & plumbagin; trifluralin & acriflavine; trifluralin & saponin; trifluralin & plumbagin and acriflavine & plumbagin combinations. These combinations were used in two doses injected in mice previously infected with *L. major* and having pentostam as reference drug. The significant decrease in the lesion sizes in the combined therapy is indicative of the occurrence of a synergistic mechanism between the drug combinations. It is interesting to note that the combined administration of saponin and other test drugs significantly ($p < 0.05$) reduced footpad size and concomitantly significantly inhibited parasite growth. This is attributable to the main biological activity ascribed to saponins: their membrane permeabilizing property. Saponin enhanced the uptake of the other drugs, thus, increasing their activity against the parasites.

There was a remarkable long-term *in vivo* activity of trifluralin, acriflavine, saponin and plumbagin at 1.25 mg/kg dosage administered subcutaneously against established *L. major* infection in BALB/c mice. The optimal dose used with the test compounds was 500 $\mu\text{g/ml}$. At 500 $\mu\text{g/ml}$ per kg of body weight daily for seven days showed a high short-term efficacy against recent infection of *L. major*. For the sake of completeness, since a direct extrapolation of the drug doses from mice to humans is unreliable, it should be noted that in *Leishmania* therapy the recommended therapeutic dose of pentostam on humans has been established to be 20 mg/kg of body weight/day for 20 days²⁶. The short-term efficacy of the test compounds against a *Leishmania* infection which evolved for two months prior to drug administration was demonstrated. This phase of murine cutaneous leishmaniasis caused by *L. major* corresponds to the end of the acute phase of the disease²⁷ and was characterized in this study as demonstrated by other researchers by a spontaneous decrease of the hepatic and a steady increase of the splenic parasite burden (Fig. 3)²⁸.

The amastigote loads were significantly lower in decreasing order in the spleens and livers of saponin, plumbagin, trifluralin and acriflavine-treated mice respectively than in those of the controls at all the time points tested. Moreover, the progression of the splenomegaly and the increase of the splenic load were found to be significantly lower in treated mice than in controls, demonstrating that parasite suppression and the inhibition of growth persisted for at least 7 to 8 wk after the end of the drug uptake.

Although data in this study do not provide a direct measure of the parasite killing 1-[amastigote load at the end of the treatment/amastigote load before the start of

the treatment], these show induction of effective leishmanicidal activity. The spleen is a major site of *Leishmania* multiplication in the natural infection in susceptible hosts. In BALB/c mice, the splenic parasite burden is initially quite low, but it increases steadily for at least three months, and unlike the hepatic burden, it does not decline spontaneously without treatment²⁹. The splenic efficacy of the test compounds should be emphasized, since until recently splenectomy was performed as the last recourse for cases of antimony resistant leishmaniasis. The efficacy of the test compounds in the spleen is compatible with the available data on its tissue distribution³⁰. The prolonged effect of the test compounds on parasite growth suggests a low catabolism and/or slow clearance, resulting in a long biological half-life of the drugs.

From the results of this study, it is evident that combination therapy was highly effective in both healing the lesion and eliminating the parasites from the liver and spleen at low drug concentration (500 µg/ml) as opposed to monotherapy at 1.25 mg/kg. Total elimination of the parasites was achieved after 7 days of treatment of both early and advanced infections. Furthermore, this duration of treatment was sufficient to clear the lesion of both the leishmanial parasites and the secondary infection, mainly bacterial, accompanying this disease. In monotherapy treatment, parasites reappeared at the site of inoculation many months later after the termination of treatment. On the other hand, in combination treatments, no reappearance of parasites at the site of infection ever occurred. This implies that monotherapy did not achieve total clearance of parasites, hence, the relapse. In contrast, combination therapy caused elimination of parasites in the internal organs. It can, therefore, be concluded that administration of the combination of drugs is effective and superior than individual drugs. The synergism shown with the combined drugs brings us to the concept of structure-function approach in fighting leishmaniasis. In 1993, Marion and others reported that higher efficacy may be correlated with molecular structural features of compounds under study³¹. This is the first time a novel antileishmanial activity in the plumbaginacea extract and triterpenoid saponin extract in combination with acridine and dinitroaniline herbicides has been reported.

CONCLUSION

Combination therapy with plumbaginacea extract and triterpenoid saponin extract in combination with acridine and dinitroaniline herbicides resulted in complete clearance of parasitemia from both the lesion site and internal organs (the livers and spleens) of *L. major*-infected BALB/c

mice. On the basis of these results and considered together with existing studies that have been reported on the safe doses and side effects of combination drug therapy, and its administration by the subcutaneous route, combination therapy is a promising approach for the treatment of *L. major* infection.

ACKNOWLEDGEMENTS

This study was funded by Deutsche Akademische Austauschdienst (DAAD) as part of Ph.D. Scholarship for J.A. Makwali. We wish to appreciate the support given by Moi University as the host University and Center for Biotechnology Research and Development (CBRD), KEMRI for providing the laboratory space to carry out the investigations.

REFERENCES

- Desjeux P. Leishmaniasis: Current situation and new perspectives. *Comparative Immunol Microbiol Infect Dis J* 2004; 27: 305–18.
- Bañuls A, Hide M, Prugnolle F. *Leishmania* and the leishmaniasis: A parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. *Ad Parasitol J* 2007; 64: 2–70.
- Murray HW. Clinical and experimental advances in treatment of visceral leishmaniasis. *Antimicrob Agents Chemother* 2001; 45: 2185–97.
- Melby Peter C. Vaccination against cutaneous leishmaniasis: Current status. *Am J Clin Dermatol* 2002; 3(8): 557–70.
- Mishra J, Saxena A, Singh S. Chemotherapy of leishmaniasis: Past, present and future. *Curr Med Chem J* 2007; 14: 1153–69.
- Basu MK, Lala S. Macrophage specific drug delivery in experimental leishmaniasis. *Curr Mol Med J* 2004; 4(6): 681–9.
- Chan MM, Tremer RE, Fong D. Effect of the anti-microtubule drug oryzalin on growth and differentiation of the parasitic protozoan *Leishmania mexicana*. *Differentiation* (Berlin) 1991; 46(1): 15–21.
- Chan MM, Grogl M, Chen CC, Bienen EJ, Fong D. Herbicides to curb human parasitic infections: *In vitro* and *in vivo* effects of trifluralin on the trypanosomatid protozoans. *Proc Nat Acad Sci USA* 1993; 90: 5657–61.
- Agbe A, Yielding KL. Kinetoplasts play an important role in the drug responses of *Trypanosoma brucei*. *J Parasitol* 1995; 6: 968–73.
- Simpson L. Effect of acriflavine on the kinetoplast of *Leishmania tarentolae*. Mode of action and physiological correlates of the loss of kinetoplast DNA. *J Cell Biol* 1968; 37: 660–82.
- Mahato SB, Garai S. Triterpenoid saponins. *Progr Chem Org Nat Prod* 1998; 74: 1–196.
- Gauthier C, Legault J, Pichette A. Recent progress in the synthesis of naturally occurring triterpenoid saponins. *Mini-Rev Org Chem* 2009; 6: 321–44.
- Hostettmann K, Marston A. Chemistry and pharmacology of natural products, *Saponins*. Cambridge, United Kingdom: Cambridge University Press 1995; p. 239–84.
- Plock A, Sokolowska-Kohler W, Presber W. Application of flow

- cytometry and microscopical methods to characterize the effect of herbal drugs on *Leishmania* spp. *Exp Parasitol* 2001; 97: 141–53.
15. Delmas F, Giorgio CD, Elias R, Gasquet M, Azas N, Mshvildadze V, Dekanosidze G, Kemertelidze E, Timon-David P. Antileishmanial activity of three saponins isolated from ivy, alpha-hederin, beta-hederin and hederacolchiside A1. *Planta Medica* 2000; 66: 343–7.
 16. Likhitwitayawuid K, Kaewamatawong R, Ruangrunsi N, Krungkrai J. Antimalarial naphthoquinones from *Nepenthes thorelii*. *Planta Medica* 1998; 64(3): 237–41.
 17. Didry N, Dubrevil L, Pinkas M. Activity of anthroquinonic and naphthoquinonic compounds on oral bacteria. *Pharmazie* 1994; 49: 681–3.
 18. Parimala R, Sachdanandam P. Effect of plumbagin on some glucose metabolizing enzymes studied in rats in experimental hepatoma. *Mole Cell Biochem* 1993; 12(1): 59–63.
 19. Itoigawa M, Takeya K, Furukawa H. Cardiotonic action of plumbagin on guinea-pig papillary muscle. *Planta Medica* 1991; 57(4): 317–9.
 20. Bhargava SK. Effects of plumbagin on reproductive function of male dog. *Indian J Exp Biol* 1984; 22(3): 153–6.
 21. Kitanov GM, Pashankov PP. Quantitative investigation on the dynamics of plumbagin in *Plumbago europea* L. roots and herb by HPLC. *Pharmazie* 1994; 49(6): 462–4.
 22. Sundar S, Rai M, Chakravarty J, Agarwal D, Agrawal N, Vaillant M, Olliaro P, Murray HW. New treatment approach in Indian visceral leishmaniasis: Single-dose liposomal amphotericin B followed by short-course oral miltefosine. *Clin Infect Dis J* 2008; 47: 1000–6.
 23. Nolan TJ, Farrel JP. Experimental infections of the multimammate rat (*Mastomys natalensis*) with *Leishmania donovani* and *Leishmania major*. *Am J Trop Med Hyg* 1987; 36(2): 264–9.
 24. Stauber LA, Franchino EM, Grun J. An eight-day method for screening compounds against *Leishmania donovani* in golden hamsters. *J Protozool* 1958; 5: 269–73.
 25. Idson B. Percutaneous absorption. *J Pharmaceut Sci* 1975; 64(6): 901–23.
 26. Barbara HL, Jonathan BD. Recommendations for treating leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. *Am J Trop Med Hyg* 1992; 46(3): 296–306.
 27. Bradley DJ. Genetics of susceptibility and resistance in the vertebrate host. *The leishmaniasis in biology and medicine*. London, United Kingdom : Academic Press 1987; p. 551–77.
 28. Rousseau D, Le Fichoux Y, Stien X, Suffia I, Ferrua B, Kubar J. Progression of visceral leishmaniasis due to *Leishmania infantum* in BALB/c mice is markedly slowed by prior infection with *Trichinella spiralis*. *Infect Immunol* 1997; 65: 4978–83.
 29. Croft SL, Yardley V, Coombs GH. Antiprotozoal activities of phospholipids analogues. *Mol Biochem Parasitol* 2003; 126: 165–72.
 30. Marschner N, Kotting J, Eibl H, Unger C. Distribution of hexadecaphosphocholine and octadecyl-methyl-glycerol-3-phosphocholine in rat tissues during steady-state treatment. *Cancer Chemother Pharmacol* 1992; 31: 18–22.
 31. Marion MC, Tzeng J, Emge TJ, Ho CT, Fong D. Structure-function analysis of antimicrotubule dinitroanilines against promastigotes of the parasitic protozoan *Leishmania mexicana*. *Antimicrob Agent Chemother* 1993; 37(9): 1909–13.

Correspondence to: Dr Judith A. Makwali, School of Science, Department of Biological Sciences, Chepkoleil Campus, Moi University, P.O. Box 1125, Eldoret 30100, Kenya.
E-mail: jmakwali@yahoo.com

Received: 3 May 2011

Accepted in revised form: 15 July 2012