TOXICITY AND EFFICACY OF AQUEOUS CRUDE EXTRACTS FROM *ALLIUM SATIVUM*, *CALLISTEMON CITRINUS* AND *MORINGA STENOPETALA* AGAINST *LEISHMANIA MAJOR*

By

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THE 4th INTERNATIONAL CONFERENCE AT KABARAK UNIVERSITY

DATE: 18th JULY, 2014

INTRODUCTION

(a) **The Leishmaniases:** protozoan infectious tropical diseases;

(b) **Forms:** CL, VL, MCL, cutaneous PKDL (Sudan);

(c) **Causative agent for CL in Kenya**: *L. major*

(d) **Vectors:**

- Phlebotomine sandflies (Africa);
- *Lutzomyia* sandflies (S. America)

(e) **Conventional drugs:** Pentostam; Glucantime; Amphotericin B; Pentamidine; paromomycin; Miltefosine (oral); a combination of paromomycin & pentostam.

(f) **Control:** Insecticides; repellants; avoid termite mounds, appropriate clothing, bed nets, and herbal extracts (rural areas).

STATEMENT OF THE PROBLEM

- **Leishmaniases Standard Drugs**: expensive, highly toxic, prolonged treatment and hospitalization;
- **Leishmaniasis is common in poverty stricken areas:** Costly drugs may not be readily available; hence victims opt for herbal therapy that have not been tested;
- **Drug resistance:** Delayed healing, high costs and frustrations;

JUSTIFICATION

a) Need for alternative affordable, less toxic, and readily available local drugs;

b) Search for cheap herbal substances that can cure leishmaniases in poverty stricken areas is inevitable;

HYPOTHESES

(a) The aqueous crude extracts are not efficacious against *L. major in vitro and in vivo* when compared to the standard leishmaniasis drugs;

(b) The aqueous crude extracts are not toxic against *L. major* promastigotes and vero cells *in vitro* when compared to the standard leishmania drugs;

MAIN OBJECTIVE

To determine the *in vitro* toxicity and *in vivo* efficacy of aqueous crude extracts from garlic vegetable (*Allium sativum*), African moringa tree (*Moringa stenopetala),* bottle brush tree (*Callistemon citrinus*) against *L. major.*

SPECIFIC OBJECTIVES

(a) To determine the minimum inhibitory concentration (MIC) of the crude extracts against *L. major* promastigotes;

(b) To establish the *in vitro* toxicity levels of aqueous crude extracts against *L. major* promastigotes and vero cells;

(b) To establish the *in vivo* activity of aqueous crude extracts against *L. major* in BALB/c mice;

(c) To determine the parasite burden in the spleens of groups of infected BALB/c mice that were treated with the test extracts and the controls (Leishmaniases drugs and phosphate

MATERIALS AND METHODS:

(I) GARLIC CLOVES: *ALLIUM SATIVUM* L

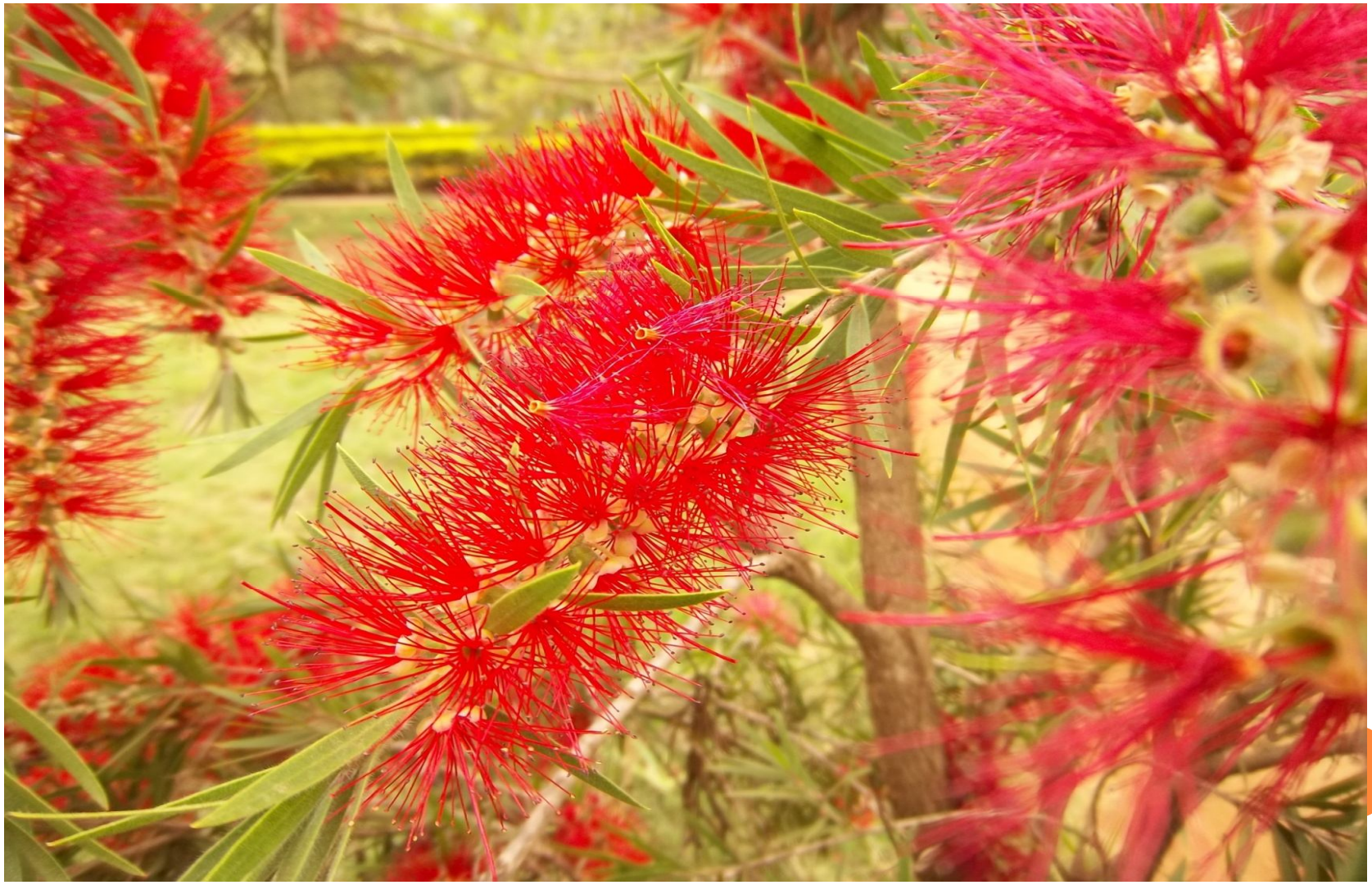
Plate 1: Bulbs and cloves of *A. satium* L.

Garlic bulbs

Garlic cloves (beans)

(II) BOTTLE BRUSH TREE FLOWERS: *CALLISTEMON CITRINUS (CURTIS) SKEELS*

Plate 2: *C. citrinus* tree.



(iii) AFRICAN MORINGA TREE: *MORINGA STENOPETALA (BAKER F)* CUFODONTIS

Plate 3: *M. stenopetala* tree and leaves

Young tree growing on slopes of Lake Baringo islands.

Young leaves on a branch

HERBAL MATERIALS

Collection: Baringo , Nakuru, Nairobi

Identification: University of Nairobi Botany Dept.

Drying: At KEMRI, room temp; brittle, constant wt.

Grinding: Powder, using electric mill;

Aqueous crude extracts:

- Extracted as described by Delahaye *et al*. 2009;

EXPERIMENTAL ANIMALS

- 6 – 8 weeks old inbred BALB/c mice;
- Obtained from ILRI and housed in KEMRI (Nairobi);

LEISHMANIA MAJOR PARASITES

Leishmania major : Strain NLB-144;

Obtained: IPR-Karen;

Culture medium: Schneider's *Drosophila* medium + 20% heat inactivated FBS + antibiotics + antifungal;

IN VITRO BIOASSAYS

Anti-Promastigote and cytotoxicity assays:

- The minimum inhibitory concentration (MIC) was determined as described by Wabwoba *et al*., 2010;
- Toxicicity levels (IC50) of extracts against promastigotes and vero cells was determined as described by Wabwoba *et al*., 2010;
- The viability of treated promastigotes and vero cells was determined as described by Mosmann formula (1983);

IN VIVO EXPTS : BALB/c MICE

(a) Infection and treatment of BALB/c mice

Footpads: inoculated subcutaneously with promastigotes;

Infected mice: treated with extracts and controls (pentostam, liposomal amphotericin B, and PBS);

Route of administration: oral, ip.

Lesions sizes: vernier caliper.

(b) Estimating parasite burden in the mice spleens

Spleen index, LDU, and total LDU were determined as described by Bradley & Kirkley (1977).

DATA ANALYSIS

- SPSS for windows at 5% level of significance.
- One way ANOVA (F test):
  - (i) Used to compare the lesion sizes in groups of mice under different treatments.
  - (ii) Compare LDU and total LDU for mice under different treatments.
  - (iii) Multiple comparisons of the individual treatments were done using both Tukey HSD and Games-Howell *post hoc* tests

RESULTS

Plant extracts yields:

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

Plant species	Part used	Code	Initial wt (g)	Yield (g)	Yield (%)
A. sativum	Bulbs	A	50	9.290	18.59
C. citrinus	Flowers	B	50	8.790	17.58
M. stenopetala	Leaves	C	50	3.200	6.40

RESULTS CONT.

Extracts toxicicity, MICs and viabilities of promastigotes:

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

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

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

RESULTS CONT.

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

Test extracts	**Code**	**IC_{50} (μg/ml)**
Aqueous:		
M. stenopetala	A	1306.68
C. citrinus	B	467.11
A. sativum	C	2105.93
Control drugs:		
Pentostam	Pento	108.58
Liposomal Amphotericin B	Amph B	60.95

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

RESULTS CONT.

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

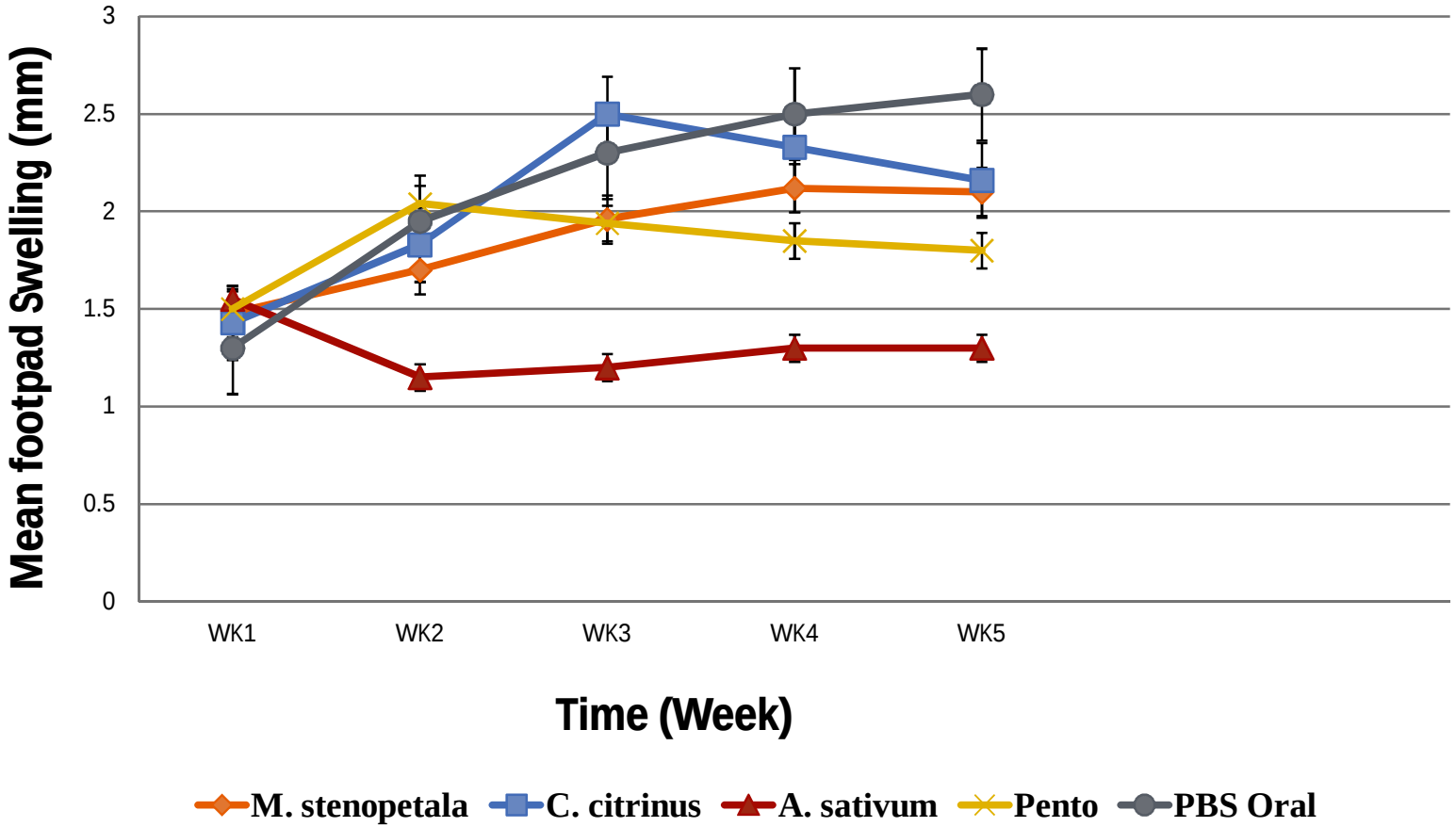


Figure 1: The foot pad swelling after oral treatment of *L. major* infected BALB/c mice with test aqueous extracts.

RESULTS CONT.

Effects of intra peritoneally (ip) administered aqueous extracts on *L. major* caused footpad lesions:

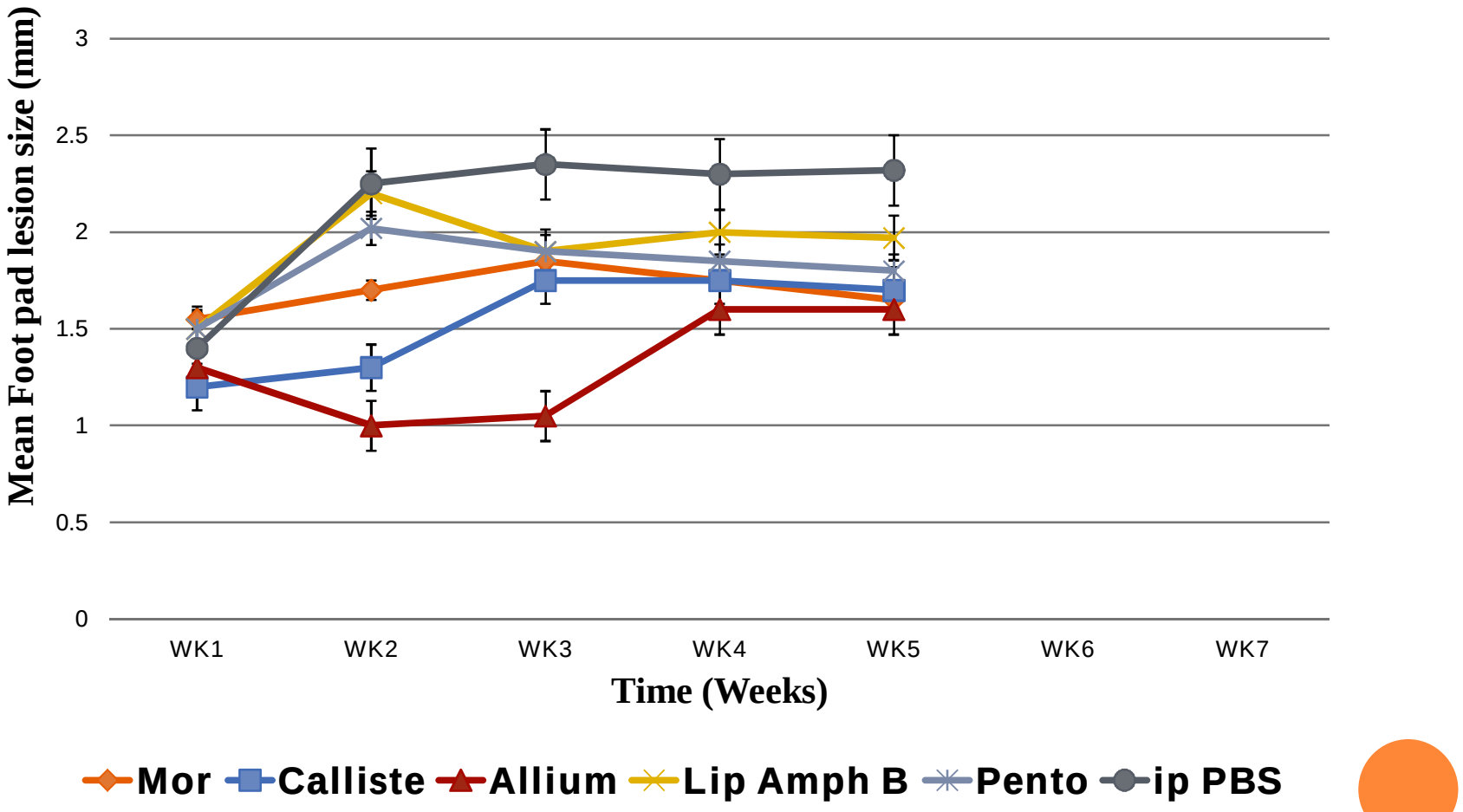


Figure 2: The foot pad swelling after intra-peritoneal treatment of *L. major* infected BALB/c mice with aqueous extracts and control drugs.

RESULTS CONT.

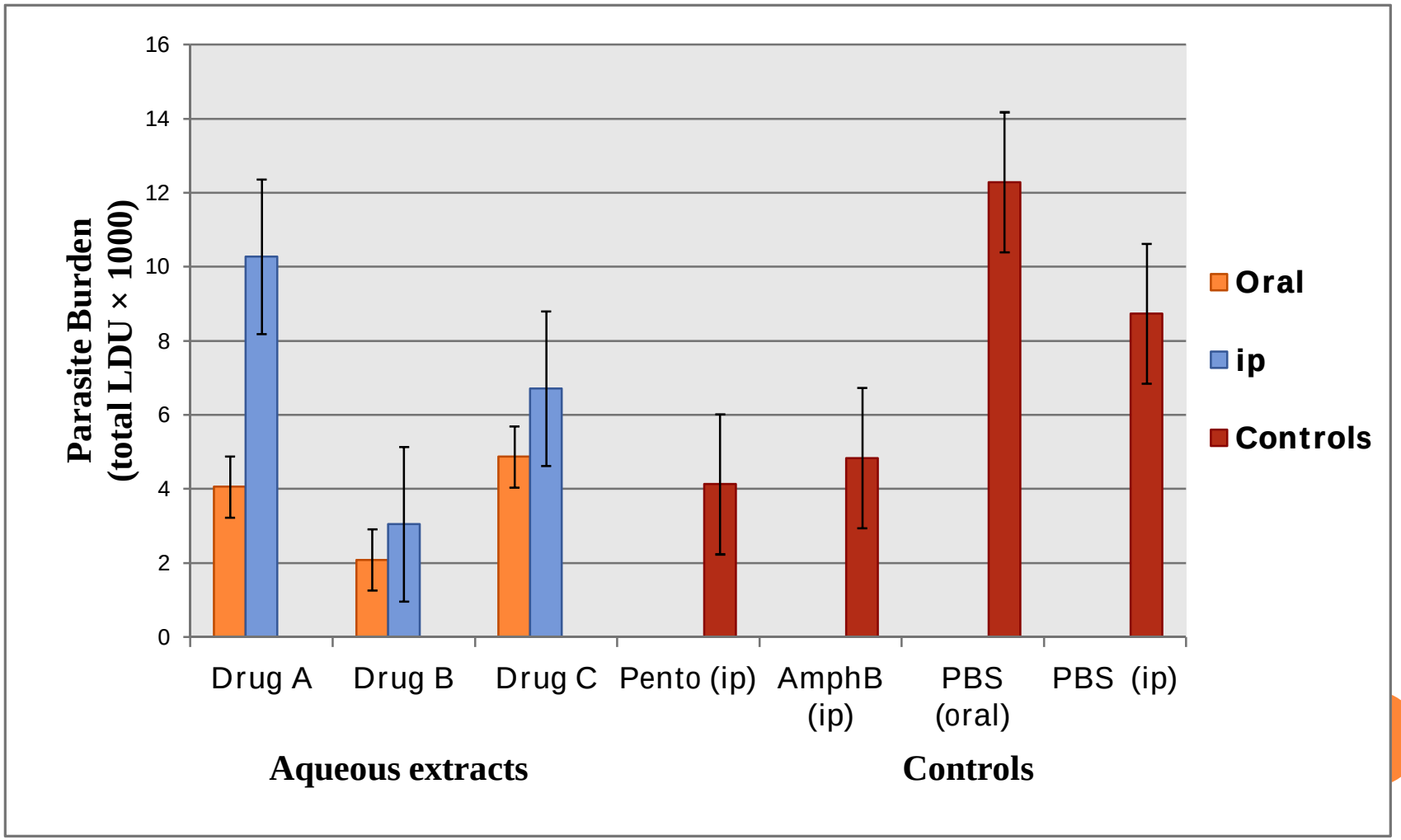
Estimation of *L. major* amastigotes in the splenocytes of treated mice

Table 4: The average spleen index ± SE, LDU ± SE and total LDU ± SE for treated infected BALB/c mice.

Test extracts & controls	Route	Ave spleen index (%)	Ave LDU	Ave total LDU (× 1000)	% parasite reduction [a]
Aqueous:					
A (*M. stenopetala*)	oral	0.51 ± 0.14	0.25 ± 0.04	4.06 ± 0.60	66.96
	ip[b]	0.54 ± 0.01	0.57 ± 0.05	10.28 ± 2.02	16.35
B (*C. citrinus*)	oral	0.71 ± 0.21	0.10 ± 0.02	2.09 ± 0.14	82.99
	ip	0.53 ± 0.03	0.19 ± 0.13	3.05 ± 2.02	75.18
C (*A. sativum*)	oral	0.42 ± 0.01	0.27 ± 0.01	4.87 ± 0.21	60.37
	ip	0.47 ± 0.05	0.33 ± 0.24	6.71 ± 4.80	45.40
Controls:					
Pentostam	ip	0.73 ± 0.19	0.18 ± 0.08	4.13 ± 1.10	66.40
Lip amph B	ip	0.61 ± 0.02	0.24 ± 0.02	4.84 ± 0.38	60.62
PBS	ip	0.54 ± 0.04	0.38 ± 0.21	8.74 ± 5.30	28.89
PBS	oral	0.56 ± 0.06	0.61 ± 0.22	12.29 ± 4.49	00.00

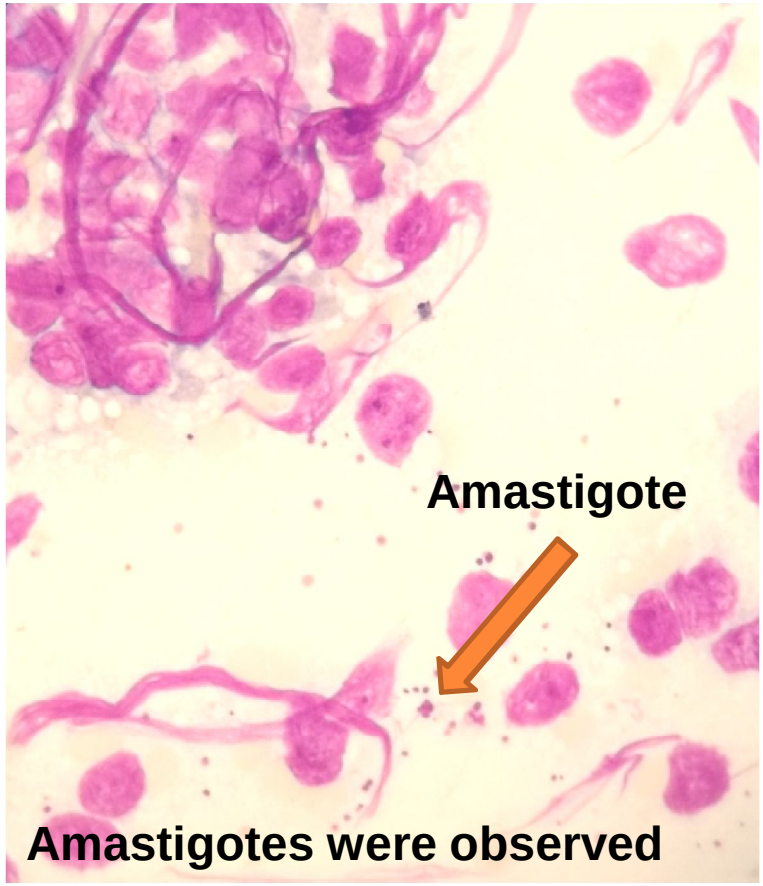
RESULTS CONT.

Figure 3: Parasite burden in spleens of *L. major* infected BALB/c mice post-treatment.

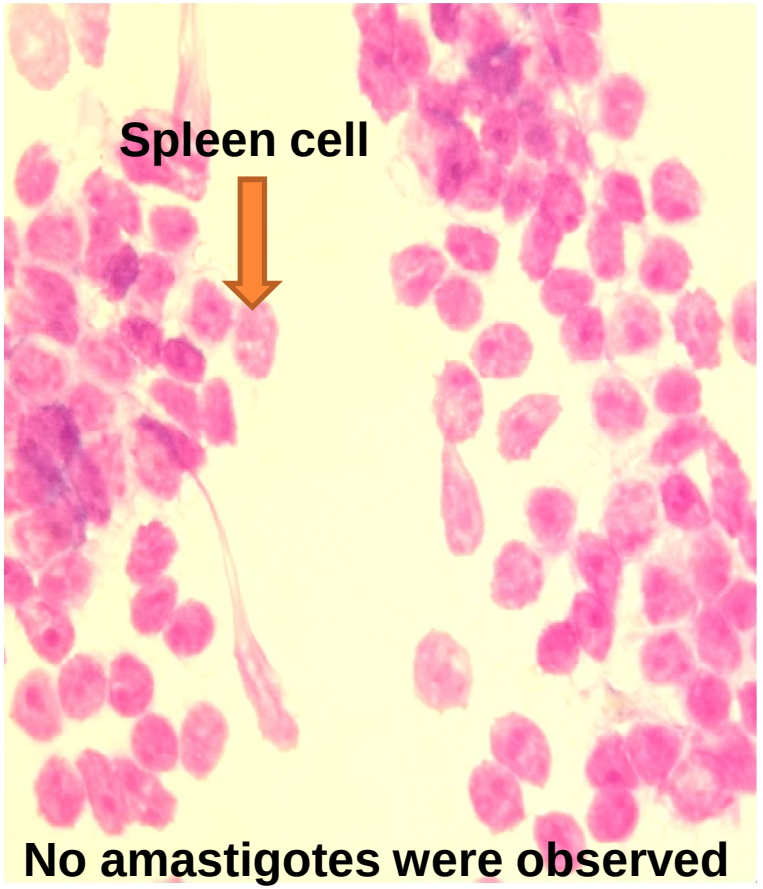


RESULTS CONT.

Splenocytes from a mouse treated with PBS orally



Splenocytes of a mouse treated with extract B oral



DISCUSSION

Efficacy of the crude aqueous extracts:

- *Allium sativum* (Garlic):
  - Active against *L. major* at 5 mg/ml *in vitro;* in line with previous studies.
  - Garlic causes apoptosis, cell shrinkage, DNA fragmentation;
  - Sulfur containing cpds like ajoene, allicin (diallyl thiosulfinate), diallyl disulfide - may have had direct inhibitory effect on the *L. major* proms;
  - Reduced the lesions in mice significantly: Garlic causes re-epithelialization of wounds and increases loosely packed collagen bundles (Ejaz *et al*., 2009).
  - Less toxic compared to pentostam and liposomal amphotericin B. (reason why garlic is used as a vegetable).
  - Being active, natural, readily available and less toxic: can be applicable to leishmaniases control.
  - The study showed that garlic had antiprotozoa activity *in vivo*: reduced the parasite burden in the spleens of infected

DISCUSSION CONT.

Callistemon citrinus (Bottle brush tree):

The aqueous extracts: -

- Oral extracts: - reduced the parasite load in the spleens significantly and this compared closely to pentostam and liposomal amphotericin B.
- Oral and ip extracts reduced the *L. major* caused lesions: - Antiprotozoa activity that reduced the amastigotes hence fast healing;
- Antibacterial activity: killing the bacteria at the wound (lesions) and quickening the healing;
- Efficacy may be attributed to essential oils (1,8 cineole and alpha pinene) that are abundant.
- Slightly toxic when compared to garlic and moringa; Toxicity attributable to the essentials oils reported to be moderately toxic.

DISCUSSION CONT.

Moringa stenopetala (African moringa tree):

- Inhibits promastigotes *in vitro* at 5 mg/ml – hence antiprotozoa activity is used as a vegetable in Ethiopia
- Low toxicity: Explains why it is used as a vegetable.
- Extracts stabilized the lesions from week three to four of treatment.
- Properties of *M. stenopetala* which could have contributed to speedy wound healing were:-
  - Rich in nutrients especially proteins needed to make new tissues.
  - Moringa is known for immune building, antiulcers and antibacterial which may speed tissue healing.
  - Moringa extracts are rich in bioactive antibiotic glucosinolates compounds (Fahay, 2005; Bellostas *et al*., 2010) -This could explained *in vivo* inhibitory activity against *L. major* amastigotes in BALB/c mice spleens, observed in the current study

CONCLUSION

- Crude aqueous extract of *M. stenopetala* ((Baker F) Cufodontis, *A. sativum* L (garlic), and *C. citrinus* (Curtis) Skeels are relatively less toxic when tested against *L. major* promastigotes and vero cells *in vitro.*
- The aqueous extracts of garlic, moringa and bottle brush were active against *L. major* promastigotes and amastigotes in the splenocytes of infected mice.
- Specifically, aqueous extracts of dry garlic significantly reduced *L. major* caused foot pad lesions in BALB/c mice when compared to phosphate buffered saline.
- Aqueous bottle brush extracts were active in decreasing the amastigotes in the spleens of infected BALB/c mice.

RECOMMENDATIONS

1. More studies on medicinal potential of aqueous crude extracts of *M. stenopetala, C. citrinus, & A. sativum* in order to get a cheaper way of controlling cutaneous leishmaniasis in poverty stricken rural areas;
2. Use of garlic in the diets should be emphasized in leishmaniasis endemic areas because as immuno-modulators they stimulate immunity to clear *Leishmania.*
3. Using *Moringa* tree as antileishmanial and as a vegetable should be emphasized in leishmaniases endemic Baringo County of Kenya, where *Moringa stenopetala* plant grows naturally.
4. Use of bottle brush tree as a herb should be promoted in Kenya. It is proving to have a broad spectrum activity.

ACKNOWLEDGEMENTS

The authors of this paper would like to thank the following:

- Scientists and researchers from KEMRI-Nbi *Leishmania* laboratory for their technical advice

- Kenyatta University and University of Nairobi for their technical advice and their active roles in this project.

THANK YOU