RESEARCH ARTICLE

In vitro study of the efficacy of *Solanum nigrum* against *Leishmania major* [version 1; peer review: 1 approved with reservations, 1 not approved]

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Abstract

Leishmania parasites (Kinetoplastida: Trypanosomatidae) are obligate intracellular parasites of macrophages that causes visceral and cutaneous leishmaniases. Currently, there is inadequate therapeutic interventions to manage this endemic tropical disease, transmitted mainly by phlebotomine sandflies hence there is need to develop affordable and effective therapeutic measures. This study determined the *in vitro* efficacy of *Solanum nigrum* methanolic and aqueous plant extracts on *Leishmania major* parasites. Cytotoxic effects of the extracts were determined using vero cells and reported as percentage viability of the cells. The promastigote parasites of *Leishmania major* were cultured and grown for 3 days in different concentrations of extracts to determine the MIC and IC₅₀ values. The *in vitro* antileishmanial efficacy was done on macrophages infected with *L. major* amastigote parasites and then treated with extracts in varying concentrations. The study revealed that all the test extracts had lower toxicity than control drugs, pentostam (IC₅₀= 0.0 92 mg/ml) and amphotericin B (IC₅₀=0.049 mg/ml). The extracts tended to show a dose dependent cytotoxic effect which corresponded to high vero cells viability as their concentration increased. Methanolic extract of *S. nigrum* from Kisii seemed to be more efficacious *in vitro* since it knocked out the promastigotes at a lower MIC level (0.5 mg/ml) when compared to all other extracts whose effective MIC level was ≥ 1 mg/ml. High concentrations of the test extracts and control drugs resulted to low infectivity and multiplication of *L. major* amastigotes. Findings from this study demonstrate that *S. nigrum* extracts have potential antileishmanial activities however; further investigation needs to be done on pure compound isolation, *in vivo* assays and clinical trials so as to use the promising compounds as effective antileishmanial agents.

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Any reports and responses or comments on the article can be found at the end of the article.		

Keywords

Leishmaniasis, Leishmania major, Solanum nigrum, Plant extracts, Toxicity

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Introduction

Leishmaniasis is a widespread disease caused *Leishmania* parasites which are transmitted by the sandfly. Desjeux (1998) reported that the disease occurred in different clinical forms, which ranged from cutaneous self-healing lesions to a fatal visceralizing form, and also included the metastasizing mucocutaneous and post-kala-azar dermal leishmaniasis. Leishmaniasis represents an important health and socioeconomic problem in 88 countries around the world, where this disease is endemic according to a study by Dujardin *et al.* (2008). Despite the death toll and disease burden of leishmaniasis, there is an acute lack of suitable therapies. Treatment of the disease depends on a limited number of drugs with limitations such as high cost, unacceptable host toxicity, poor efficacy, lack of availability, and acquired parasite drug resistance as reported in studies by Barrett & Fairlamb (1999); Fairlamb (2003) and Stuart *et al.* (2008). Therefore, the development of cheap, available, effective and less toxic drugs is of paramount importance. Medicinal plants are the best alternative since they possess natural active components that can be effective against parasitic infections or can be used in development of commercial drugs.

Several studies have reported that some plants are effective against *Leishmania* parasites both *in vitro* and *in vivo*. The findings of studies by Kinuthia *et al.* (2014); Ndeti *et al.* (2016) and Njau *et al.* (2016) revealed that plants such as *Allium sativum*, *Callistemon citrinus*, *Moringa stenopetala* and *Aloe secundiflora* were effective against *Leishmania major* parasites. A study by Wabwoba *et al.* (2010) indicated that a combination of *M. stenopetala* with *Allium sativum* induced apoptotic effect in *Leishmania major* promastigotes. Similar results were reported in studies by McClure *et al.* (1996) where the growth of *Leishmania mexicana* and *Leishmania chagasi* were inhibited by *A. sativum*, and by Khademvatan *et al.* (2011) where *A. sativum* extract induced apoptosis in *Leishmania major* parasites. Schlein *et al.* (2001) reported that *Ricinus communis* (Malpighiales: Euphorbiaceae) possessed anti-leishmanial effects both in *Phlebotomus dubosqi* (Diptera: Psychodidae), the vector for *L. major* in Israel, and in *in vivo* in BALB/c mice when used alone, as revealed in a study by Oketch *et al.* (2006), or in combination with *Azadiracta indica* (Sapindales: Meliaceae) as indicated by Jumba *et al.* (2015).

The findings of some studies have shown that Solanaceae family plants have medicinal effects against various parasitic infections. The findings of Laban *et al.* (2015) showed that *Solanum aculeastrum* was effective against *Leishmania major* parasites. Additionally, Mishra *et al.* (2013) reported that a prenyloxy-naphthoquinone obtained from roots of *Plumbago zeylanica* (Caryophyllales: Plumbaginaceae) has anti-leishmanial activity against *Leishmania donovani*. There was a significant difference between the EC₅₀ for the isolated compound and miltefosine, the standard drug (P< 0.001) against *L. donovani* promastigotes and amastigotes. Studies on *L. major* in Kenya by Makwali *et al.* (2015) have shown that *Plumbago capensis* possess anti-leishmanial effects. The current study evaluated the *in vitro* anti-leishmanial activity of *Solanum nigrum* extracts on *L. major* parasites.

Methods

Ethical considerations

The proposal for this research work was submitted to the KEMRI Scientific Steering Committee (SSC), for approval and was given ethical clearance (Number: KEMRI/SSC-2028) on the use of the mice as the animal model by the Ethical Review Committee (ERC). All experimental animals at the end of the experiment were sacrificed by injection of 100 µl sodium pentobarbital and disposed of according to the regulations of Animal Care and Use (ACUC) through incineration.

Experimental design

The *in vitro* studies were carried out using a comparative study design. Pentostam (Glaxo Operations (UK) Limited, Barnard Castle, UK) and amphotericin B (AmBisome®; Gilead, Foster City, CA, USA) were used as the standard drugs to compare their efficacy and toxicity with those of the test extracts. RPMI-1640 and Schneider's *Drosophila* media (Thermo Fisher Scientific, Waltham, Massachusetts, USA) were used as the control in *in vitro* experimental chemotherapeutic studies.

Plant collection and preparation of the extracts

Fresh leaves of *Solanum nigrum* were collected from Kisii and Bungoma, Kenya, where the plant is abundant. The plants were transferred to the Center of Traditional Medicine and Drug Research (CTMDR) at KEMRI (Nairobi, Kenya) and dried at 25°C until they became brittle and attained a constant weight. The dried plants were separately ground using an electric mill (Christy & Norris Ltd., Chelmsford, England) into powder followed by extraction using water and analytical grade methanol. The methanolic extracts were prepared as described by Mekonnen *et al.* (1999) and Cock (2012). Immediately, 100 g of ground plant material was soaked in 500 ml of analytical grade methanol for 72 h at room temperature with gentle shaking. The mixture was filtered using Whatman No.1 filter papers and concentrated using a rotary evaporator to obtain dry methanolic extracts. The extracts were coded as A and B for methanolic extracts of *S. nigrum* (Bungoma) and *S. nigrum* (Kisii), respectively. The aqueous extracts were prepared as described by Delahaye *et al.* (2009). Briefly, 100 g of the dried ground plant material in 600 ml of distilled water was placed in a water bath at 70°C for 1.5 h. The mixture was filtered using Whatman No.1 filter papers and then the filtrate frozen, dried and weighed. The extracts were coded as C and D for *S. nigrum* (Kisii) and *S. nigrum* (Bungoma), respectively. The extracts were then stored at 4°C until required for bioassays.

Mice and *Leishmania* parasites

A total of four 8-week-old male inbred BALB/c mice with weights that ranged between 25 and 29 g were obtained from KEMRI. There were eight BALB/c mice per cage in the animal house kept at 23–25°C under a 12/12 h light/dark cycle and were fed on standard diet in the form of mouse pellets and given tap water *ad libitum*. The mice were handled in accordance with the regulations set by the Animal Care and Use Committee at KEMRI. The mice were used for extraction of peritoneal macrophages that were used for anti-amastigote assay.

The *Leishmania major* strain (IDUB/KE/94=NLB-144) which was originally isolated in 1983 from a female *Phlebotomus dubosqi* collected from Marigat, Baringo County in Kenya were used. The parasites were grown to stationary phase at 25°C in Schneider's *Drosophila* medium supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Hyclone® USA), 100 U/ml penicillin and 500 µg/ml streptomycin (Hendricks & Wright, 1979), and 250 µg/ml 5-fluorocytosine arabinoside (Kimber *et al.*, 1981). The stationary-phase metacyclic stage promastigotes were then harvested by centrifugation at 1500g for 15 min at 4°C. The metacyclic promastigotes were then used for the *in vitro* assays.

Preparation of the stock solutions of test extracts

Stock solutions of the crude plant extracts were made in Schneider's *Drosophila* culture medium for anti-leishmanial assays and filtered through 0.22-µm filter flasks in a laminar flow hood (Biological Safety Cabinet). The stock solutions were then stored at 4°C and retrieved later for both *in vitro* bioassays.

Cytotoxicity assay using Vero cells to determine IC₅₀ values

This assay was used to test the cytotoxicity of individual extracts against Vero cells (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and the results were presented as IC₅₀ values. The assay was carried out as described by Wabwoba *et al.* (2010). Vero cells were grown in minimum essential medium (MEM) (ATCC® 30-2003™) supplemented with 10% FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) in 25 ml cell culture flasks incubated at 37°C in a humidified 5% CO₂ for 24 h. The Vero cells were harvested by trypsinization and pooled in 50-ml centrifuge tubes from which 100 µl of cell suspension was moved into two wells of rows A-H in a 96-well flat-bottomed microtiter plate at a concentration of 1×10⁶ cells per ml of the culture medium per well and incubated at 37°C in 5% CO₂. The MEM was gently aspirated off and 150 µl of the test extracts (A, B, C and D) were added at concentrations of 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml and 31.25 µg/ml in the micro titer plates. The plates containing the Vero cells and test extracts were further incubated at 37°C for 48 h in a humidified 5% CO₂ atmosphere. The controls wells comprised of Vero cells and medium while the blank wells had medium alone. A total of 10 µl of (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reagent were added into each well and incubated further for 2–4 h until a purple precipitate (formazan) was visible under the microscope. The media together with MTT reagent was gently be 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 microtiter plate reader at wavelength of 570 nm. The IC₅₀ values of the extracts were determined automatically using the Chemosen program 2.

Evaluation of minimum inhibitory concentration (MIC)

The MICs were determined as described by Wabwoba *et al.* (2010). Briefly, the *L. major* metacyclic promastigotes at concentration of 1×10⁶ promastigotes per ml of the culture medium were treated with individual methanolic test extracts A and B

whose concentrations were 2000 µg/ml, 1000 µg/ml, 500 µg/ml and 250 µg/ml. These test procedures were repeated for aqueous extracts C and D. The lowest concentration of the individual test extracts in which no live promastigotes were observed was the MIC.

Evaluation of IC₅₀ and antipromastigote assay

Metacyclic promastigotes at a concentration of 1×10⁶ promastigotes per ml of the culture medium were grown for 48 h in 24-well microtiter plates at 25°C. Aliquots of the promastigotes were transferred into 96 well microtiter plates and incubated further at 27°C for 24 h after which 200 µl of the highest concentrations (2 mg/ml) of the individual test extracts were added before serial dilutions of 2.0×10³, 1.0×10³, 5.0×10², 2.5×10², 1.25×10², and 6.25×10¹ were carried out. The control wells contained *L. major* promastigotes in culture medium alone whereas the blank wells had the culture medium alone. The plates were incubated further at 27°C for 48 h and 10 µl of MTT reagent was into each well and incubated further for 4 h. The medium and MTT reagent were aspirated off the wells. Next, in each well, 100 µl of DMSO was added and the plates shaken for 5 minutes. Absorbances were read at 562 nm using a microtiter reader. The absorbance readings were used to generate IC₅₀ values for the different plant extracts using the Chemosen program v2.

Survival of *L. major* promastigotes was stratified as follows: +++, 75–100% survival compared with control; ++, 50–<75% survival compared with control; +, 25–<50% survival compared with control; -, <25% survival compared with control; -, absence of live promastigotes.

Anti-amastigote assay

The anti-amastigote assay was carried out as described by Delorenzi *et al.* (2001). The peritoneal macrophages were obtained from four clean BALB/c mice. The mice were anaesthetized using 100 µl pentobarbital sodium (Sagatal®). The body surface of the mouse was disinfected with 70% ethanol after which it was opened dorso-ventrally to expose the peritoneum. A total of 10 µl sterile cold PBS was injected into the peritoneum. After injection, the peritoneum was gently massaged for 2 min to dislodge and release macrophages into the PBS. The peritoneal macrophages were then be harvested by withdrawing the PBS. The PBS containing the macrophages was washed through centrifugation at 2,000g for 10 min and the pellet obtained was re-suspended in RPMI-1640 culture medium. The macrophages were adsorbed in 24-well plates for 4 h at 37°C in 5% CO₂. Non-adherent cells were washed away with cold sterile PBS and the adherent macrophages were incubated overnight in RPMI-1640 culture medium. Adherent macrophages were then infected with *L. major* promastigotes and were further incubated at 37°C in 5% for 4 h, after which they were washed with sterile PBS to remove the free promastigotes, which were not engulfed by the macrophages. This was followed by incubation of the preparation for 24 h in RPMI-1640 culture medium.

Pentostam and liposomal amphotericin B at concentrations of 125 µg/ml, 250 µg/ml and 500 µg/ml were used as positive

control drugs to compare the parasite inhibition with that by plant extracts. The medium and test extracts or drug was replenished daily for 3 days. After 5 days, the macrophages were washed with sterile PBS at 37°C, fixed in methanol and stained with 10% Giemsa. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and the count was expressed as infection rate (IR) and multiplication index (MI) as described by Berman & Lee (1984) in the calculations below:

IR (%) = Number of infected macrophages per 100 macrophages

$$MI(\%) = \frac{\text{Number of amastigotes in experimental culture per 100 macrophages}}{\text{Number of amastigotes in control culture per 100 macrophages}} \times 100$$

Statistical analysis

The IC₅₀ values were determined using Chemosen program v2. Data for infection rates and multiplication indices were saved as percentages and then were analyzed using SPSS 13.0 programme. The results were expressed as mean values ± standard deviation (SD). Statistical analysis were done using one way ANOVA and Tukey's post hoc test and *p* values < 0.05 were considered significant.

Results

Cytotoxicity assays using Vero cells

Generally, all the test extracts studied were less toxic (i.e. higher IC₅₀ values) to Vero cells when compared to the control drugs, pentostam (0.03 mg/ml) and amphotericin B (0.01 mg/ml) (Table 1).

The methanolic and aqueous extracts tended to show a dose-dependent cytotoxic effect which corresponded to low IC₅₀ values as their concentration increased. Methanolic extracts of *S.nigrum* from Bungoma and Kisii had IC₅₀ values of 0.57 mg/ml and 0.50 mg/ml while those their aqueous counterparts were 0.76 mg/ml and 0.64 mg/ml (Table 1).

The survival of *L. major* promastigotes after exposure to extracts and controls

Preliminary studies involved exposure of the *L. major* promastigotes to extracts and control drugs at varying concentrations *in vitro*. The *L. major* parasites cultured in Schneider's *Drosophila* medium were taken as the negative control because the parasites continued to multiply. Both pentostam and amphotericin B used were able to inhibit the *Leishmania major* promastigotes growth at an MIC of 31.25 µg/ml. The Schneider's *Drosophila* medium, on the other hand, led to maximum survival of *L. major* promastigotes (+++++) (Table 2).

Both aqueous extracts of *S. nigrum* from Bungoma and Kisii (C and D) inhibited the survival of *Leishmania major* promastigotes at an MIC of 2000 µg/ml. *S. nigrum* methanolic extracts from Bungoma (A) lowered *L. major* parasites at a concentration of 1000 µg/ml, while that from Kisii (B) inhibited *L. major* multiplication at an MIC of 500 µg/ml.

The concentrations of the extracts that were effective against *L. major* promastigotes *in vitro* were high (>0.5 mg/ml) compared to those of the standard drugs (0.03125 mg/ml). The efficacy of methanolic extracts was better than their respective aqueous counter parts (Table 2).

Table 1. The IC₅₀ values showing the effects of the extracts and controls on Vero cells. The initial concentration of the test extracts was 1000 µg/ml while that of control drugs was 500 µg/ml before serial dilution.

Code	Test extracts and drugs	IC ₅₀ , mg/ml
A	<i>S. nigrum</i> (Bungoma) methanolic extract	0.57
B	<i>S. nigrum</i> (Kisii) methanolic extract	0.50
C	<i>S. nigrum</i> (Kisii) aqueous extract	0.64
D	<i>S. nigrum</i> (Bungoma) aqueous extract	0.76
Pento	Pentostam	0.03
Ampho B	Amphotericin B	0.01

Table 2. Survival of *L. major* promastigotes in varying concentrations of the plant extracts as observed under a light microscope.

Test extracts and drugs	Extraction type	Code	Concentration of the extracts (µg/ml)			
			2000	1000	500	250
<i>S. nigrum</i> (Bungoma)	Methanolic	A	-	+	++	++++
<i>S. nigrum</i> (Kisii)	Methanolic	B	-	-	-	++
<i>S. nigrum</i> (Kisii)	Aqueous	C	+	+++	++++	++++
<i>S. nigrum</i> (Bungoma)	Aqueous	D	+	+++	++++	++++
Control drug			Standard drug concentration, µg/ml			
			125	62.5	31.25	15.625
Pentostam			-	-	-	++
Amphotericin B			-	-	-	+
Schneider's <i>Drosophila</i> Medium			++++	++++	++++	++++

++++, 75–100% survival compared with control; +++, 50–<75% survival compared with control; ++, 25–<50% survival compared with control; +, <25% survival compared with control; -, absence of live promastigotes.

Minimum inhibitory concentrations (MIC) and IC₅₀ values of the extracts on *L. major* promastigotes

The MICs of *S. nigrum* from Bungoma (A) and *S. nigrum* from Kisii (B) methanolic extracts were 1000 µg/ml and 500 µg/ml, respectively. Both aqueous extracts of *S. nigrum* from Kisii (C) and *S. nigrum* from Bungoma (D) had MIC values of 2000 µg/ml. The standard drugs had lower MICs (31.25 µg/ml) against *L. major* promastigotes compared to the test extracts. Schneider's *Drosophila* medium, which was the negative control, supported maximum survival of *Leishmania major* promastigotes (Table 3).

IC₅₀ values determined indicated the effectiveness of the test extracts or the controls in inhibiting the promastigotes by 50%. Pentostam and amphotericin B had IC₅₀ values of 0.08 mg/ml and 0.04 mg/ml, respectively, while *S. nigrum* from Bungoma (A) and *S. nigrum* from Kisii (B) methanolic extracts had IC₅₀ values of 1.81 mg/ml and 1.28 mg/ml, respectively. Similarly, aqueous extracts of *S. nigrum* from Kisii (C) and *S. nigrum* from Bungoma (D) had IC₅₀ values of 1.18 mg/ml and 1.84 mg/ml, respectively. Based on the IC₅₀ values, the aqueous extract of *S. nigrum* from Kisii (C) was the most effective of the extracts (Table 3).

For extracts that shared the same MICs, higher IC₅₀ values tended to correspond to increased *in vitro* survival of promastigotes of *Leishmania*. The methanolic extract of *S. nigrum* from Kisii seemed to be more efficacious *in vitro* since it knocked out the promastigotes at a lower MIC level (0.5 mg/ml) when compared to all other extracts whose effective MIC level was ≥1 mg/ml (Table 3).

Anti-amastigote assay (macrophage assay)

RPMI-1640 medium with no drug had an infection rate of 96.7% (Table 4), which implied that it supported maximum growth of *Leishmania major* amastigotes in peritoneal macrophages (Figure 1). The leishmaniasis drugs pentostam and liposomal amphotericin B inhibited the *in vitro* survival of *L. major* amastigotes, corresponding to low infection rates of 26.3% and 21.0%, respectively, at a concentration of 50 µg/ml (Table 4).

At a concentration of 125 µg/ml, the methanolic extracts of *S. nigrum* from Bungoma (A) and *S. nigrum* from Kisii (B) had

infection rates of 71.0±2.3% and 68.0±2.7%, respectively. Similarly, the infection rates of aqueous extracts, *S. nigrum* from Kisii (C) and *S. nigrum* from Bungoma (D) were 78.0±2.5% and 85.3±1.2% (Table 4).

The methanolic extract of *S. nigrum* from Kisii (B) inhibited the survival of *L. major* amastigotes better than the other extracts in all the concentrations studied (Figure 2). High concentrations of the test extracts and control drugs resulted in low IRs and MIs of *L. major* amastigotes. The efficacies were dose-dependent. The difference between the IRs of test extracts and the control drugs were statistically significant ($P < 0.05$).

When the MIs of amastigotes in peritoneal macrophages treated with 125 µg/ml of methanolic test extracts (A and B) were compared with those treated with 50 µg/ml of amphotericin or pentostam, using one-way ANOVA, there was a statistically significant difference ($P < 0.001$). A Tukey *post hoc* test revealed that the MI of methanolic extracts of *S. nigrum* from Kisii (A and B) at 500 µg/ml were statistically significant from that of pentostam and amphotericin B ($P = 0.001$).

When the infection rates of methanolic extracts of 500 µg/ml A and B were compared with those of amphotericin B using Tukey's *post hoc* test, the difference in each case was statistically significant, ($P < 0.001$ and $P = 0.001$, respectively). Comparisons of the IRs for extracts C and D with those of amphotericin B or pentostam followed a similar trend, where Tukey's *post hoc* test indicated a significant difference ($P < 0.05$) for each comparison. The MIs of pentostam and amphotericin B were not statistically different ($P \geq 0.05$) at a concentration of 25 µg/ml.

Dataset 1. Raw data for absorbance values from MTT assay and subsequent calculation of IC50 values on Vero cells for extracts of *Solanum nigrum* from Kisii and controls

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For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain indicated test samples, with extract concentrations of 31.25, 62.5, 125, 250 µg/ml, 500 and 1000 µg/ml, respectively, and control drug concentrations of 16.125, 31.25, 62.5, 125, 250 and 500 µg/ml, respectively.

Table 3. MIC and IC₅₀ levels of test extracts on *L. major* promastigotes. The extracts concentration ranged from 2000 µg/ml to 250 µg/ml for MIC and 62.5 µg/ml for IC₅₀ determination, while the concentration of the positive controls ranged from 125 µg/ml to 15.625 µg/ml for MIC and 500 µg/ml to 15.625 µg/ml for IC₅₀ determination.

Code	Test extracts and drugs	MIC, mg/ml	IC ₅₀ , mg/ml
A	Methanolic <i>S. nigrum</i> (Bungoma)	1	1.81
B	Methanolic <i>S. nigrum</i> (Kisii)	0.5	1.28
C	Aqueous <i>S. nigrum</i> (Kisii)	2	1.18
D	Aqueous <i>S. nigrum</i> (Bungoma)	2	1.84
Pento	Pentostam	0.03125	0.08
Amph B	Amphotericin B	0.03125	0.04

Table 4. Infectivity of *L. major* amastigotes in macrophages after treatment with various test extracts and standard drugs.

Code	Concentration, µg/ml	Infection rate, %	Multiplication index, %
A	500	55.6±2.7	60.3±3.4
	250	63.6±4.6	65.5±5.1
	125	71.0±2.3	71.8±0.8
B	500	51.6±4.3	59.2±2.5
	250	59.0±5.0	67.7±3.6
	125	68.0±2.6	69.3±2.5
C	500	69.6±4.1	67.1±3.2
	250	74.6±5.0	74.1±3.8
	125	78.0±2.5	83.3±2.9
D	500	76.6±4.1	79.7±3.0
	250	78.6±4.4	82.3±2.5
	125	85.3±1.2	89.2±0.5
Pentostam	100	21.3±2.3	21.8±3.0
	50	26.3±1.2	25.7±3.7
	25	26.7±2.3	34.4±2.8
Amphotericin B	100	19.0±2.3	18.8±2.2
	50	21.0±2.1	27.2±2.5
	25	22.0±1.5	33.7±1.8
RPMI-1640	96.67± 0.9		100.0

Figure 1. Showing a BALB/c mice peritoneal macrophage having engulfed *L. major* amastigotes at 5 days post infection in RPMI-1640 medium culture. Am= amastigote, Nuc= Nucleus, and Cyt= Cytoplasm.

Figure 2. The *in vitro* activities of the test extracts and controls against *L. major* amastigotes in BALB/c peritoneal macrophages.

Dataset 2. Raw data for absorbance values from MTT assay and subsequent calculation of IC50 values (on Vero cells) for extracts of *Solanum nigrum* from Bungoma

<https://doi.org/10.5256/f1000research.15826.d214922>

For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain test samples, with extract concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/ml, respectively.

Dataset 4. Raw data for absorbance values from MTT assay and subsequent calculation of IC50 values (on promastigotes) for extracts of *Solanum nigrum* from Bungoma

<https://doi.org/10.5256/f1000research.15826.d214924>

For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain test samples, with extract concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg/ml, respectively.

Dataset 3. Raw data for absorbance values from MTT assay and subsequent calculation of IC50 values (on promastigotes) for extracts of *Solanum nigrum* from Kisii and controls

<https://doi.org/10.5256/f1000research.15826.d214923>

For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain test samples, with extract concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg/ml, respectively C to H for extract samples and 16.125, 31.25, 62.5, 125, 250 and 500 µg/ml, respectively, for standard drugs.

Dataset 5. Anti-amastigote (macrophage) assays

<https://doi.org/10.5256/f1000research.15826.d214929>

Discussion

This study has shown that *S. nigrum* has anti-leishmanial activity against *Leishmania* parasites. The results indicated that the plant extracts of *S. nigrum* obtained from Kisii and Bungoma have the potential to inhibit *L. major* promastigotes *in vitro*. The current study further established that the concentrations

of the extracts that were effective (MIC) against *L. major* promastigotes *in vitro* were relatively high (>0.5 mg/ml), as compared to those of pentostam and amphotericin B, which both inhibited the promastigotes at 0.03125 mg/ml. The efficacy of methanolic extracts was better than their respective aqueous counterparts. Schneider's *Drosophila* medium was used as a negative control and supported maximum survival of the *L. major* promastigotes *in vitro*. This was expected because this medium supports the growth of *Leishmania* promastigotes and amastigotes, as described by Hendricks & Wright, 1979. The efficacy of test extracts was higher than that of Schneider's *Drosophila* medium. The slight differences that have been noted between the two allopatric plants could be due to factors such as difference in the presence and composition of the phytochemicals; a study by Aritho *et al.* (2017) on *T. vogelii* also revealed such differences.

A study by Son *et al.* (2003) showed that extracts from *S. nigrum* leaves had the potential to be used in treatment of tumors, especially liver cancer, and also used for treatment of lung cancer, bladder and gastric carcinoma as indicated by studies done by Mueller *et al.* (2005) and Ashwani *et al.* (2012). Additionally, studies by Jain *et al.* (2011) and Ashwani *et al.* (2012) revealed that methanol crude extracts obtained from *Solanum nigrum* possessed antioxidant activity due to its DPPH radical scavenging activity.

Studies by Estevez *et al.* (2007); Filho *et al.* (2013); Hubert *et al.* (2014); and Shen *et al.* (2012) revealed that some species of *Solanum* had antileishmanial activity. Findings from the study by Estevez *et al.* (2007) showed that the extracts of *S. stramonifolium* had activity against *L. amazonensis* amastigotes. This activity was attributed to steroid derivatives which include cistol A and steroidal alkaloids, which form the main components in *Solanum* species (Abreu Miranda *et al.*, 2013; Filho *et al.*, 2013).

Cytotoxic assays using Vero cells showed that the test extracts were less toxic compared to the standard antileishmanial drugs. Generally, the increase in the dose of the extracts led to a higher cytotoxic effect on *L. major* promastigotes, resulting in inhibition of the growth of the parasites. Many drugs used for treatment of leishmaniasis are highly toxic (Santos *et al.*, 2008) and this study confirmed that pentostam and amphotericin B are toxic compared with the extracts tested. The continued use of the contemporary leishmaniasis drugs despite their toxicity is mainly due to lack of an alternative. The use of herbal medicine can be a cheaper and available alternative. The aqueous extracts of both *S. nigrum* from Bungoma and Kisii (IC_{50} , 0.76 mg/ml and 0.64 mg/ml, respectively) were less toxic than methanolic extracts (IC_{50} of 0.57 mg/ml and 0.50 mg/ml, respectively).

The lower the toxicity of the test extracts, the higher the viability of Vero cells after exposure to extracts and vice versa. According to Das *et al.* (2007) the *Solanaceae* family plants have been reported to be poisonous both to humans and livestock. Their toxicity has been attributed to the presence of tropane

alkaloids, which when ingested in large quantities, causes anticholinergic effects. Another study by Glossman-Mitnik (2007) reported that the toxicity of *S. nigrum* which is edible is due to solanine, a glycoalkaloid which causes toxicity as the concentration increases.

The *S. nigrum* from Bungoma (A) and *S. nigrum* from Kisii (B) methanolic crude extracts had infection rates of $71.0 \pm 2.3\%$ and $68.0 \pm 2.6\%$, respectively, at a concentration of 125 μ g/ml. Similarly, the infection rates of aqueous extracts, *S. nigrum* from Kisii (C) and *S. nigrum* from Bungoma (D) were $78.0 \pm 2.5\%$ and $85.3 \pm 1.2\%$, respectively. In comparison, the leishmaniasis drugs, pentostam and liposomal amphotericin B inhibited the *in vitro* survival of *L. major* amastigotes more effectively and this corresponded to low infection rates of 26.3% and 21%, respectively, at a concentration of 50 μ g/ml. There was a significant difference between the efficacy of the test extracts and that of the *Leishmania* drugs ($P < 0.05$). This observation indicates that *S. nigrum* extracts which are known for their antimicrobial and antifungal potential (Abbas *et al.*, 2014; Musto, 2014). When the test extracts were compared with the controls, IR of macrophages by *L. major* amastigotes in plain RPMI-1640 medium (negative control) was $96.7 \pm 0.9\%$. This agrees with Berman & Wyler (1980) who observed that the amastigotes of *Leishmania tropica* and *Leishmania donovani* in peritoneal macrophages multiplied about three fold in six days when grown in RPMI-1640 medium in absence of antileishmanial agents. The trend was similar for MIs. This observation was similar to that by Wabwoba *et al.* (2010), who observed that the IRs of amphotericin B and pentostam at 100 μ g/ml were 9.0% and 11%, respectively. In this study, however, although the difference between MI for amphotericin B and pentostam at 50 μ g/ml was not statistically significant, the *in vitro* efficacy of amphotericin B in suppressing the amastigotes multiplication was higher than that of pentostam.

Conclusion

The findings of this study have justified the claimed medicinal importance of *Solanum nigrum* as a remedy for various infections. It can be concluded that the crude extracts of *S. nigrum* possess considerable anti-leishmanial activity, especially against *Leishmania major*, which were used in this study. The plant may contain potent anti-parasitic compounds, effective in the treatment of *Leishmania* infections. However, further investigation needs to be conducted on pure compound isolation, toxicological studies and clinical trials so as to use the promising compounds as effective antileishmanial agents.

Data availability

Dataset 1. Raw data for absorbance values from MTT assay and subsequent calculation of IC_{50} values on Vero cells for extracts of *Solanum nigrum* from Kisii and controls. For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain indicated test samples, with extract concentrations of 31.25, 62.5, 125, 250 μ g/ml, 500 and 1000 μ g/ml, respectively, and control drug concentrations of 16.125, 31.25, 62.5, 125,

250 and 500 µg/ml, respectively. DOI: <https://doi.org/10.5256/f1000research.15826.d214921> (Mutoro *et al.*, 2018).

Dataset 2. Raw data for absorbance values from MTT assay and subsequent calculation of IC₅₀ values (on Vero cells) for extracts of *Solanum nigrum* from Bungoma. For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain test samples, with extract concentrations of 31.25, 62.5, 125, 250, 500 and 1000 µg/ml, respectively. DOI: <https://doi.org/10.5256/f1000research.15826.d214922> (Mutoro *et al.*, 2018).

Dataset 3. Raw data for absorbance values from MTT assay and subsequent calculation of IC₅₀ values (on promastigotes) for extracts of *Solanum nigrum* from Kisii and controls. For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain test samples, with extract concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg/ml, respectively C to H for extract samples and 16.125, 31.25, 62.5, 125, 250 and 500 µg/ml,

respectively, for standard drugs. DOI: <https://doi.org/10.5256/f1000research.15826.d214923> (Mutoro *et al.*, 2018).

Dataset 4. Raw data for absorbance values from MTT assay and subsequent calculation of IC₅₀ values (on promastigotes) for extracts of *Solanum nigrum* from Bungoma. For sorted raw absorbance data, columns 3, 6, 9 and 12 contain untreated cells; wells A1, A2, A4, A5, A7, A8, A10, A11, B1, B2, B4, B5, B7, B8, B10 and B11 contain medium only. Rows C-H contain test samples, with extract concentrations of 62.5, 125, 250, 500, 1000 and 2000 µg/ml, respectively. DOI: <https://doi.org/10.5256/f1000research.15826.d214924> (Mutoro *et al.*, 2018).

Dataset 5. Anti-amastigote (macrophage) assays. DOI: <https://doi.org/10.5256/f1000research.15826.d214929> (Mutoro *et al.*, 2018).

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Comments:

Abstract:

Abstract should be rewritten even the IC 50 of standard drugs are not matching with the result section of the manuscript.

Keywords: *Leishmania major* and *Solanum nigrum* should be in italics throughout the manuscript; eg: *Leishmania major* and *Solanum nigrum*

Methods:

No subheading or paragraph describes the maintenance of cell lines which is being used for cytotoxicity. This is important information which is missing. Please add these details.

Mice and *Leishmania* parasites:

We wonder that the parasites are being cultured in *Drosophila* medium instead of M199 or RPMI. *Drosophila* medium is often used to achieve the fast primary isolation rather than maintenance. The M199 and RPMI media are far better for the maintenance of the parasites.

Preparation of the stock solution of test extracts:

The stock solution of the extracts in the culture medium should be prepared freshly immediate before the use. It should not be stored for later use as it may change the pH value of the medium which can hamper the activity of the extracts. It also can lead to the high rate of contamination. Furthermore, if at all, it should be stored only in the solvent not in the medium.

cytotoxicity assay using Vero cells to determine IC 50:

This paragraph needs to be rewritten. There are no details of counting of the cells.

In the beginning of the manuscript, the authors mention volume of the suspension of cells from which 100ul of cells were directly used and finally declared it as 1×10^6 cells per ml. To our minds the centrifugation and counting of the cells with its procedure is extremely important to claim any activity of anti leishmanial compounds. Otherwise results are difficult to be reproduced.

It is also not clear, how the authors could test 6 dilutions of 4 extracts?. As per the description of plate designing the cells were plated only in two wells of A-H row? (as mentioned in line no 11 in the above subheading). This totals to only 16. It is serious concern for this reviewer.

Again authors have not mentioned the time duration of incubation before aspirating out the MEM medium. It is the required time for the adhering to the solid phase. Otherwise cell will also be aspirated off with the medium, and making the study claims untenable.

Again the same section authors have no-where mentioned the standard drugs and concentrations thereof, if at all used for the assay. But in the result section the results of the standard drugs are mentioned on the top. How come?

Authors should also mention the exact timing of incubation performed by them after MTT addition, not 2-4 hrs, which is very arbitrary and not acceptable.

Evaluation of MIC & Evaluation of IC 50 and anti-promastigote assay.

A big question is, if the authors performed two different tests to evaluate MIC and IC 50 value individually? If not then the heading can be merged as Antipromastigote assay to evaluate the IC 50 and MIC of the Test extracts. Evaluation of IC 50 and antipromastigote assay.

What does authors mean by serial dilutions of 2.0×10^3 , 1.0×10^3 , 5.0×10^2 , 2.5×10^2 , 1.25×10^2 , and 6.25×10^1 ? The dilutions are written in different manner, either as log or serial, but not like this. If these are extract's dilutions then there should be uniformity in the unit representation of the concentration of the drugs for all assays.

Somewhere it is mg/ml and somewhere its ug/ml for the same drugs.

Again there is no discussion of standard drugs under this subheading.

Authors should mention method of visual reading by microscopic observation (if done) before mentioning the survival comparison.

Anti-amastigote assay:

The amount of sterile cold PBS injected in the peritoneum seems very low in volume. 10 ul will go nowhere and no one can withdraw it back. Write carefully.

What was the ratio of macrophage and promastigotes used for this assay. No counting and ratio is mentioned.

Were 4 hrs sufficient for the infection incubation as the promastigotes takes at least 8-12 hrs for engulfment to Maximum 24 hrs. In the manuscript again the plate was incubated for 24 hrs but after the washing step. After reading their protocol, we really cannot believe the results, if these

are genuine. And what was the need of second incubation. In this section the concentration of plant extracts used are missing.

Authors must have mentioned the magnification (x) used for the microscopic observation.

Amastigotes are only possible to visualize at 1000 x magnification with oil which is not possible in 24 well plate directly. We can't understand how authors could count the amastigotes.

Statistical analysis:

All the experiments could have been performed in duplicate wells in the same assay. And also could have been repeated thrice for mean and SD Value, which is not done in cytotoxic and antipromastigote assays mentioned in this manuscript. Culture was done in duplicate only in anti-amastigote assay, for which p value can be calculated by simple way. ANOVA and Tukey's Test is not needed.

Results:

As there are so many flaws in methodology section there is no meaning of evaluating-the results. At every step the results must match with the methodology used. This is missing in this otherwise poorly written paper.

Even the concentration unit is not uniform. The IC 50 value of standard drugs are not even appropriate .

Figure 1 is not even clear to visualize the macrophages. How come author counted the amastigotes?

Recommendation: The authors are encouraged to go through a recent publication by Srivastava et al, 2017¹ and resubmit the manuscript as fresh.

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1. Srivastava S, Mishra J, Gupta A, Singh A, et al.: Laboratory confirmed miltefosine resistant cases of visceral leishmaniasis from India. *Parasites & Vectors*. 2017; **10** (1). [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

No

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Reviewer Report 31 August 2018

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Abstract

- Grammar: "Parasites cause" not "parasites causes".
- There are currently inadequate therapeutic interventions to manage this endemic tropical disease, transmitted mainly by phlebotomine sandflies hence there is need to develop affordable and effective therapeutic measures.
- The current study sought to determine the *in vitro* efficacy of *Solanum nigrum* methanolic and aqueous plant extracts on *Leishmania major* parasites.
- When making reference to numbers use digit if more than ten i.e. "three" and not "3".
- There should be an indication of the country where the study was conducted, how the plants for plant extracts were collected and the animal models used.
- There is no indication of how the results of the study were analyzed statistically.
- Methanolic extract of *S. nigrum* from Kisii seemed to be more efficacious *in vitro* since it knocked out the promastigotes at a lower MIC level (0.5 mg/ml) when compared to all other extracts whose effective MIC level was ≥ 1 mg/ml.
- Last sentence needs rephrasing.

Introduction

- Grammar, especially some words lacking in connectives i.e. in first line the word "by" is missing between "caused" and "Leishmania".
- Leishmaniasis represents an important health and socioeconomic problem in 88 countries around the world, where this disease is endemic according to a study by (Dujardin et al. 2008¹).
- There are long sentences which need to be broken to make sense.

- There is need for the authors to demonstrate the need for investigating the plants they used given that a number of studies show promising results. What was different with the plant?

Methods

- The mice were handled in accordance with the regulations set by the Animal Care and Use Committee at KEMRI.
- Statistical analyses should be specifically in respect of the assays conducted rather than general presentation.
- How were survival rates of *L. major* promastigotes analyzed?
- Why did the authors not use same concentrations of the test extract and control drugs then increase concentrations of test extracts if required?
- How was Tukeys test used?
- Scientific names should be italicized in this section and the rest of the manuscript.
- Remove repetitions of the methods in this section.
- Titles of tables should not be combined with what should be footnotes i.e. table 1 and 3.
- Rework on table 2 for better presentation
- Avoid use of abbreviation IRs, not standard.
- Figure 1 not necessary.

Discussion

- Avoid direct repetition of results in discussion i.e. most of paragraph 1.
- Authors should insert the explanation for why there are differences in extracts after the relevant sentence.
- What is the relevance of the second paragraph to the current study?
- Discuss each section of the results separately and thoroughly by comparing with other studies, including own opinions and explanations.

References

- Are the phrases in red font after each reference a journal requirement? If not delete.
- Datasets sites should be removed from reference section.

References

1. Dujardin JC, Campino L, Cañavate C, Dedet JP, et al.: Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. *Emerg Infect Dis.* 2008; **14** (7): 1013-8 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

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