Efficacy of the combination of crude extracts of *Solanum nigrum* and *Plumbago capensis* on *Leishmania major* [version 1; peer review: 1 approved, 1 approved with reservations]

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**Abstract**

**Background:** Leishmaniasis is an endemic tropical disease caused by *Leishmania* parasites, transmitted mainly by phlebotomine sandflies, impacting both health and socioeconomic wellbeing. Currently there are inadequate therapeutic measures to manage the disease thus indicating the need for the development of affordable and effective therapeutic interventions from herbal plants as alternative medicine. This study investigated the *in vitro* antileishmanial effects of blends of crude extracts of *Solanum nigrum* and *Plumbago capensis* against *Leishmania major*.

**Methods:** The promastigote parasites of *Leishmania major* were cultured and grown for 3 days in different concentrations of the individual extracts to determine minimum inhibitory concentrations (MIC). The *in vitro* antileishmanial efficacy was determined by exposing promastigotes and macrophages infected with *L. major* to the blends of extracts in ratios of 2000:250, 1000:500, 500:1000 and 250:2000. Finally, nitric oxide released by *L. major* infected macrophages that were treated with the plant extracts at ratio of 125:125 was quantified using a standard nitrite curve.

**Results:** The individual methanol extracts were most effective in inhibiting the growth of promastigotes with MIC values between 0.25 mg/ml and 1.0 mg/ml as compared to aqueous extracts. The most active ratios for the blends were 250:2000 and 2000:250 for methanolic and aqueous blends respectively. The infection rates and multiplication indices associated with all the combined extracts were significantly different (*P*< 0.05) from those of pentostam and amphotericin B at all the concentrations studied. The OD for the combined test extracts ranged between 0.034 and 0.041 and these corresponded to < 5 µM of NO.

**Conclusion:** Findings from this study demonstrate that combination therapy using *S. nigrum* and *P. capensis* extracts is effective in treating
Leishmania major infection. Based on our findings we recommend in vivo studies to be conducted to determine the efficacy of these combined therapies against Leishmania major.

**Keywords**
Leishmaniasis, Leishmania, Combined, Plumbago capensis, Solanum nigrum

This article is included in the Neglected Tropical Diseases collection.

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Introduction

Leishmaniasis are diseases caused by Leishmania parasites transmitted by the bite of female Phlebotominae sand flies (Piscopo & Mallia, 2006). Desjeux, (2004) indicates that 350 million people are at risk globally, 12 million people are infected with Leishmania parasites and that as many as 2 million new cases occur each year in over 80 Countries.

Studies conducted by Abreu Miranda et al. (2013); de Carvalho & Ferreira (2001) indicates that screening of plant extracts and plant derived compounds is an effective therapy for leishmaniasis that avoids exposure to potentially toxic drugs. The World Health Organisation (WHO) (2006), reported that the most pressing research needs for Leishmania control are the search for alternative and cheap drugs for oral, parenteral (injections) or topical administration in shorter treatment cycles, and identification of mechanisms to facilitate access to existing control measures, including health sector reform in some developing countries.

After increasing unresponsiveness to most of the monotherapy regimens, combination therapy has found new scope in the treatment of leishmaniasis. The findings of Jha et al. (2005) indicated that the combination of antileishmanial drugs could reduce the potential toxic side effects, prevent drug resistance and increase their efficacy in conjunction. Firooz et al. (2006) and Mishra et al. (2011) reported the superiority of the combination of paromomycin with other drugs for the treatment of visceral leishmaniasis. Studies by Jha et al. (2005) and Firooz et al. (2006) which evaluated combined chemotherapy against visceral leishmaniasis in Kenya using oral allopurinol and endogenous pentostam demonstrated the superiority of the combined drugs. Research shows that natural libraries of plant compounds with recognized antiparasitic activities can be screened and used in development of antileishmanial compounds. This study investigated the effect of combining crude extracts of Solanum nigrum and Plumbago capensis on Leishmania major parasites in vitro.

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 sagatal 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 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 (0° 40' 49.7352" S and 34° 46' 37.4196" E), where the plant is abundant. Plumbago capensis whose activity has been established (Makwali et al., 2015) was collected from Upper Hill area of Nairobi County, Kenya (1°17'59.0"S, 36°48'58.0"E) The plants were transferred to the Center of Traditional Medicine and Drug Research (CTMDR) at KEMRI (Nairobi, Kenya) and dried at 25°C in the shade until they became brittle and attained a constant weight. The dried plants were separately ground using an electric mill (Christy & Norris Ltd., Chelmsford, England, model 8) into powder followed by extraction using water and analytical grade methanol (Sigma, 82762). The methanolic extracts were prepared as described by Mutoro et al. (2018a). 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 (Sigma, Z240079) and concentrated using a rotary evaporator (Cole-Parmer - RE400) to obtain dry methanolic extracts. The extracts were coded as A, B and C for methanolic extracts of S. nigrum (Bungoma), S. nigrum (Kisii) and P. capensis, respectively. The aqueous extracts were also prepared as described by Mutoro et al. (2018a). 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 was frozen, dried and weighed. The extracts were coded as D, E and F for P. capensis, 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 six 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 (Orchid BREEDER, UK) 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 and quantification of nitric oxide produced by macrophages treated with blends of extracts.

The Leishmania major strain (IDUB/KE/94=NLB-144) which was originally isolated in 1983 from a female Phlebotomus dubosi collected from Marigat, Baringo County in Kenya were used. The parasites were grown to stationary phase at 25°C in Schneider’s Drosophila medium (Fisher Scientific, 21720024) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Hyclone® USA, SH30071031H), 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 promastigotes were then harvested by centrifugation at 1500g for 15 min at 4°C (Thermo Fisher Scientific 75004061 mySPIN 6 Mini Centrifuge, 1189M94EA). 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 as described by Mutoro et al. (2018a). Briefly, plant extracts were made in Schneider’s Drosophila culture medium (Fisher Scientific, 21720024) 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.

Evaluation of minimum inhibitory concentration (MIC) and active ratios of test extracts and blends on L. major promastigotes
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 and aqueous test extracts whose concentrations were 2000 μg/ml, 1000 μg/ml, 500 μg/ml and 250 μg/ml. Similarly, the promastigotes were treated with combined extracts in fixed ratios of 2000:250, 1000:500, 500:1000 and 250:2000. The L. major promastigotes treated with the single extracts and the blends were stained with 100 μL of trypan blue dye while on a microscope slide and observed under the light microscope (XSZ-107 Series Biological Microscope, Sam-Tech Diagnostics) to check their motility and viability compared to the Schneider’s Drosophila medium as the negative control. The lowest concentration of the individual test plant extracts and the blends in which no live promastigotes were observed was the MIC and active ratio for the individual test extracts and the blends respectively.

Anti-amastigote assay
The anti-amastigote assay was carried out as described by Mutoro et al. (2018a). The peritoneal macrophages were obtained from 4 clean BALB/c mice. The mice were anaesthetized using 100μl pentobarbitone sodium (Sagatal®; Sigma, P3761). The body surface of the mouse was disinfected with 70% ethanol after which it was torn dorso-ventrally to expose the peritoneum. 10μl of sterile cold phosphate buffered saline (PBS) was injected into the peritoneum. After injection, the peritoneum was gently massaged for 2 minutes to dislodge and release macrophages into the PBS. The peritoneal macrophages were then harvested by withdrawing the PBS. The PBS containing the macrophages was washed through centrifugation at 2,000g for 10 minutes and the pellet obtained was re-suspended in RPMI-1640 culture medium. The macrophages were adsorbed in 24-well plates for 4 hours at 37°C in 5% CO₂. Non-adherent cells were washed with cold sterile PBS and the adherent macrophages were incubated overnight in RPMI culture medium. Adherent macrophages were then infected with L. major promastigotes and were further incubated at 37°C in 5% CO₂ for 4 hours 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 hours in RPMI-1640 culture medium. The infected macrophages were then treated with combinations of both aqueous and methanolic extracts at fixed ratios of 500:125, 125:125 and 125:500. Pentostam and liposomal amphotericin B were used as positive control drugs to compare the parasite inhibition with that of blends of plant extracts. The medium and blends of 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 (Thermo Scientific™, 9990715). The number of amastigotes were determined by counting microscopically at least 100 infected macrophages in triplicate cultures, and the count was expressed as infection rate (IR) and multiplication index (MI) as described by Berman & Lee, (1984) in the formulas below;

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

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

Determination of nitric oxide production
Measurement of nitric oxide (NO) production was carried out as described by Gamboa-Leon et al. (2007). BALB/c mice peritoneal macrophages at a concentration of 1x10⁶ cells per culture medium were placed in each well in 96-well microtiter plates and allowed to adhere at 37°C in 5% CO₂ humidified atmosphere. 2 hours later, the peritoneal macrophages were incubated further in RPMI -1640 medium with 10% FBS for 48 hours in presence of blends of aqueous and methanolic test extracts and the controls. At least 100 μl of macrophage culture supernatants were collected and frozen until when they were required for NO measurement. NO was measured using the Greiss reaction for nitrates (NO₂) as described by Hollzmuller et al., 2002. NO₂ is one of the products released when the breakdown of NO occurs in the macrophages. NO in the collected supernatants is therefore estimated by quantifying the NO₂ content. A nitrite standard reference curve was prepared by dispensing 50 μl of RPMI-1640 with 10% FBS into wells in rows B-H of the first 3 columns in a 96 well plate. 100µM sodium nitrite solution were added to the remaining 3 wells in row A of a 96-well micro titer plate, and immediately a six serial two fold dilutions (50 μl/well) were performed in triplicate columns 1, 2 and 3 down the plate to generate a curve that corresponded to the concentrations 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 μM.

Secondly, 50 μl of the sample supernatant from the wells with macrophages treated with blends of test extracts were added into the wells in triplicate at fixed ratio of 125:125. Greiss reagent A (Fisher Scientific, G7921) were dispensed to all the experimental samples and into the wells containing sodium nitrite solution. Following an incubation of 5 minutes at room temperature, 50 μl of Greiss reagent B (Fisher Scientific, G7921) in water were dispensed into all the wells and incubated for a further 5 minutes at room temperature before measuring the optical density (OD) of the purple/magenta azo compound at 520 nm using micro titer reader (PerkinElmer). The values for the standard nitrite for each of the blend of extracts were read from the standard curve plotted, by reading the NO concentration that corresponds to the absorbance values of the samples.

Statistical analysis
The 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
Minimum inhibitory concentrations (MIC) of individual test extracts on *L. major* promastigotes

MICs for single extracts and active ratios for the blends of the extracts were detected by looking at the motility and viability of the parasites in the wells as compared to the Schneider’s *Drosophila* medium (SIM) as the negative control (Table 1 and Table 2).

The MICs of the individual methanolic extracts, *S. nigrum* from Bungoma (A), *S. nigrum* from Kisii (B) and *P. capensis* (C) against *L. major* promastigotes were 1mg/ml, 0.5 mg/ml and 0.25 mg/ml respectively. The MICs of the individual aqueous extracts, *P. capensis* (D), *S. nigrum* from Kisii (E) and *S. nigrum* from Bungoma (F) were 0.5 mg/ml, 2 mg/ml and 2 mg/ml respectively. In comparison, the MICs of pentostam and amphotericin B against *L. major* promastigotes were both 0.03125 µg/ml. The Schneider’s *Drosophila* medium supported the survival of *L. major* promastigotes to the maximum (Table 3).

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

The survival levels of promastigotes after treatment with blends of extracts were determined by comparing with survival in both the positive controls (Pentostam and Amphotericin B) and negative control (Schneider’s *Drosophila* medium). A blend of *S. nigrum* (Bungoma) and *P. capensis* (AC) and *S. nigrum* (Kisii) and *P. capensis* (BC) methanolic extracts in a ratio 250:2000 inhibited the *L. major* promastigotes in vitro 100% while ratios 500:1000 and 1000:500 decreased the promastigotes to minimum survival (25%) levels (+) and moderate (50%) levels (++) respectively. Ratio 2000:250 supported the survival of promastigotes to highly moderate (75%) levels (+++) when compared with the controls. A blend of *S. nigrum* from Bungoma and Kisii (AB) methanolic extracts was efficacious in inhibiting the parasites growth to minimum levels (+) at ratio 1:8 and there was moderate growth at ratios 1000:500 and 500:1000 (Table 4).

A blend of the aqueous extracts of *P. capensis* and *S. nigrum* from Kisii (DE) at ratio of 2000:250 led to complete inhibition of parasites growth while ratios 1000:500, 500:1000 and 250:2000 supported growth of the parasites. A blend of aqueous extracts of *P. capensis* and *S. nigrum* from Bungoma (DF) at ratio 2000:250 inhibited the growth of the parasites to minimum levels (+) and a blend of *S. nigrum* from Kisii and Bungoma (EF) supported growth of the parasites at all ratios (Table 4).

Both pentostam and amphotericin B drugs were able to inhibit the growth of *L. major* promastigotes in vitro at a concentration of 31.25 µg/ml. The Schneider’s *Drosophila* medium, on the

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**Table 1.** The set up for minimum inhibitory concentration (MIC) determination for the single extracts.

| 2mg/ml | 1mg/ml | 0.5mg/ml | 0.25mg/ml | 2mg/ml | 1mg/ml | 0.5mg/ml | 0.25mg/ml |
|--------|--------|----------|-----------|--------|--------|----------|-----------|
| A      |        |          |           |        |        |          |           |
| B      |        |          |           |        |        |          |           |
| C      |        |          |           |        |        |          |           |

**Table 2.** The set up for determination of the active ratios for the blends of extracts.

| 2000 µg/ml | 1000 µg/ml | 500 µg/ml | 250 µg/ml | 2000 µg/ml | 1000 µg/ml | 500 µg/ml | 250 µg/ml |
|------------|------------|-----------|-----------|------------|------------|-----------|-----------|
| A          |            |           |           | D          |            |           |           |
| B          |            |           |           | E          |            |           |           |
| A          |            |           |           | D          |            |           |           |
| C          |            |           |           |            |           |           |           |
| B          |            |           |           | E          |            |           |           |
| C          |            |           |           |            |           |           |           |
Table 3. The minimum inhibitory concentration (MIC) levels of *Leishmania major* promastigotes following treatment with individual test extracts and controls. The extracts concentration ranged from 2000 µg/ml to 250 µg/ml, while the concentration of the positive controls ranged from 125 µg/ml to 15.625 µg/ml.

| Test extracts and drugs | Code | MIC (mg/ml) |
|-------------------------|------|-------------|
| Methanolic              |      |             |
| *Solanum nigrum* (Bungoma) methanolic extract | A | 1 |
| *Solanum nigrum* (Kisii) methanolic extract | B | 0.5 |
| *Plumbago capensis* methanolic extract | C | 0.25 |
| Aqueous                 |      |             |
| *Plumbago capensis* water extract | D | 0.5 |
| *Solanum nigrum* (Kisii) water extract | E | 2 |
| *Solanum nigrum* (Bungoma) water extract | F | 2 |
| Pentostam               | Pento | 0.03125 |
| Amphotericin B          | Ampho B | 0.03125 |

Table 4. Survival of *Leishmania major* promastigotes in varying ratios of blends of test extracts and different concentrations of controls (standard drugs) as observed under a light microscope. ++++ indicates maximum (100%) survival, +++ shows highly moderate (75%) survival, ++ shows moderate (50%) survival, + shows minimum (25%) survival and – indicates absence of detectable and live promastigotes when compared to both the positive and negative controls.

| Code | Blends and Controls | Ratio of the extracts based on mic<sup>a</sup> |
|------|---------------------|---------------------------------------------|
|      |                     | 2000:250 1000:500 500:1000 250:2000          |
| A:B  | *S. nigrum* (Bungoma): *S. nigrum* (Kisii) methanolic extracts | ++++ ++ ++ + |
| A:C  | *S. nigrum* (Bungoma): *P. capensis* methanolic extracts | +++ ++ + - |
| B:C  | *S. nigrum* (Kisii): *P. capensis* methanolic extracts | +++ + + - |
| D:E  | *P. capensis*: *S. nigrum* (Kisii) water extracts | - ++ +++ +++ |
| D:F  | *P. capensis*: *S. nigrum* (Bungoma) water extracts | + ++ ++++ +++ |
| E:F  | *S. nigrum* (Kisii): *S. nigrum* (Bungoma) water extracts | ++ +++ +++ +++ |

| Controls | Concentrations of standard drugs (µg/ml) |
|----------|-----------------------------------------|
|          | 125  | 62.5 | 31.25 | 15.625 |
| Pentostam| Pento | -    | -     | ++     |
| Amphotericin B | Amph B | -    | -     | +      |
| Schneider’s Insect Medium | SIM | ++++ | ++++ | ++++ |

<sup>a</sup> mic = minimum inhibitory concentration which indicated the level at which the extract was inhibiting the promastigotes. *S. nigrum* - *Solanum nigrum*, *P. capensis* - *Plumbago capensis*

other hand, supported the maximum (100%) growth survival of *L. major* promastigotes as indicated by four pluses (++++) when compared to the positive controls, pentostam and amphotericin B (Table 4).

Anti-amastigote assay (Macrophage assay)
When the methanolic extracts were combined in ratios of 500:125, 125:125 and 125:500, the blend of *S. nigrum* from Kisii and *P. capensis* (BC) had the lowest infection rate of 46.7% at a con-
centration ratio of 125:500. Similarly, the methanolic extracts combinations of *S. nigrum* from Bungoma and Kisii (AB) and *S. nigrum* from Kisii and *P. capensis* (AC) resulted to infection rate (IR) of 61.7% at the same ratio (Table 5).

Combinations of the aqueous extracts of *P. capensis* and *S. nigrum* from Kisii (DE), *P. capensis* and *S. nigrum* from Bungoma (DF) and *S. nigrum* from Kisii and Bungoma (EF) at the ratio of 125:500 resulted to infection rates of 70.0%, 78.0% and 78.7% respectively. The efficacy of combined aqueous extracts *S. nigrum* from Kisii and Bungoma (EF) in the ratio of 125:500, in inhibiting the infectivity and multiplication of *L. major* amastigotes in BALB/c peritoneal macrophages *in vitro* was lower than all the other blends of extracts (Figure 1 and Table 5). The blend of methanolic extract of *S. nigrum* from Kisii and *P. capensis* (BC) performed the best with the multiplication index (MI) of 50.6% at the ratio of 125:500. RPMI 1640 medium which had no drug incorporated supported the growth of *L. major* amastigotes in peritoneal macrophages (Figure 2) more effectively and this was indicated by a high infection rate (IR) of 89.7% (Table 5). In contrast, 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 21.0 % respectively at a concentration of 50 µg/ml (Table 4). The IRs and MIs associated with all the combined extracts were significantly different (< 0.05) from those of pentostam and amphotericin B at all the concentrations studied.

### Determination of nitric oxide production

Nitric oxide (NO) plays a key role as a leishmanicidal effector molecule in host macrophages (Gamboa-Leon et al., 2007). Therefore the effect of the blends of test extracts on NO production was evaluated *in vitro*. BALB/c mice peritoneal macrophages were incubated in RPMI-1640 medium for 48 hours in the presence or in absence of blends of test extracts. In order to determine the amount of NO triggered by the combined extracts, their optical densities (absorbencies) were determined using Griess Reagent system. All the absorbencies for the combined extracts ranged between 0.034 to 0.041 (Table 6). These OD corresponded to < 5 µM of NO in the standard nitrite curve for the blends of both the methanolic and aqueous extracts. RPMI-1640 medium produced similar negligible levels of NO (Table 7).

### Discussion

Natural products which have been found to possess antileishmanial activities can provoke alternative treatment for antimonial-resistant *Leishmania* strains (Monzote, 2009). In cases where the infectious agent fails to respond to single therapy, combined therapy is often adopted. Studies by Melaku et al. (2007); Nyakundi et al. (1994) and Sundar et al., 2008) on leishmaniasises reported that antileishmanial drugs combination improved their efficacy and reduced resistance, the dosage required and toxicity levels.

As observed in the current study, the standard drugs were significantly more effective (P ≤ 0.05) against *Leishmania* promastigotes and amastigotes as compared to all the blends of the extracts. It was observed in the present study that all the blends of extracts induced little (< 1.5 µM) production of NO by peritoneal macrophages which might have played a role in the amastigote inhibition.

Freitas-Junior et al. (2012), study demonstrated that miltefosine and amphotericin B or paromomycin combinations were effective against antimony-resistant VL infections. A study

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**Table 5. The *in vitro* infection rates (IR) and multiplication indices (MI) of Leishmania major amastigotes per 100 BALB/c mice peritoneal macrophages following treatment with blends of test extracts and controls.**

| Treatment (Blends +controls) | Ratio | Infection rates (%) | Multiplication indices (%) |
|-----------------------------|-------|---------------------|---------------------------|
| A:B                         | 500:125 | 72.3±3.528  | 73.9±4.598    |
|                             | 250:250 | 69.0±2.646  | 68.4±2.318    |
|                             | 125:500 | 61.7±3.844  | 67.6±4.104    |
| A:C                         | 500:125 | 65.0±2.309  | 68.2±3.609    |
|                             | 250:250 | 62.7±1.763  | 64.6±2.653    |
|                             | 125:500 | 61.7±3.756  | 56.9±2.717    |
| B:C                         | 500:125 | 57.0±2.887  | 55.7±3.159    |
|                             | 250:250 | 55.7±3.528  | 52.1±3.593    |
|                             | 125:500 | 46.7±4.256  | 50.6±3.291    |
| D:E                         | 500:125 | 55.3±2.333  | 61.1±2.627    |
|                             | 250:250 | 57.7±2.028  | 65.2±3.150    |
|                             | 125:500 | 70.0±2.309  | 70.4±3.729    |
| D:F                         | 500:125 | 64.3±4.055  | 62.6±0.736    |
|                             | 250:250 | 64.7±2.333  | 69.0±2.338    |
|                             | 125:500 | 78.0±3.214  | 70.8±2.685    |
| E:F                         | 500:125 | 68.3±3.283  | 74.2±1.488    |
|                             | 250:250 | 70.7±2.906  | 77.6±4.693    |
|                             | 125:500 | 78.7±2.603  | 81.8±3.946    |
| Pentostam                   | 100    | 21.3±2.333  | 21.8±2.922    |
|                             | 50     | 26.3±1.202  | 25.7±3.704    |
|                             | 25     | 26.7±2.333  | 34.4±2.829    |
| Amphotericin B              | 100    | 19.0±1.527  | 18.8±2.196    |
|                             | 50     | 21.0±2.082  | 27.3±2.488    |
|                             | 25     | 22.0±2.309  | 33.7±1.780    |
| RPMI                        | 89.67±3.180 | 100%          |
Figure 1. *In vitro* activities of blends of extracts at ratios of 500:125, 125:125 and 125:500 as represented by infection rates of *Leishmania major* amastigotes in treated BALB/c mice peritoneal macrophages.

Figure 2. *In vitro* activities for controls (pentostam and amphotericin B) as represented by infection rates of *Leishmania major* amastigotes in treated BALB/c mice peritoneal macrophages.
Table 6. Nitric oxide concentration (µM), Absorbance (OD) units of the standard nitrite (positive control) and RPMI (negative control).

| NO (µM) | Standard Nitrite (Positive control) | RPMI (Negative control) |
|---------|-------------------------------------|-------------------------|
| 0       | 0.021±0.002                         | 0.028±0.000             |
| 1.5625  | 0.047±0.001                         |                         |
| 3.125   | 0.076±0.002                         |                         |
| 6.25    | 0.113±0.001                         |                         |
| 12.5    | 0.160±0.003                         |                         |
| 25.0    | 0.266±0.006                         |                         |
| 50.0    | 0.505±0.02                          |                         |
| 100.0   | 0.982±0.003                         |                         |

Table 7. Nitric oxide production by the BALB/c mice peritoneal macrophages after being stimulated by blends of both aqueous and methanolic extracts at ratio of 125:125.

| Code | Blends and drugs                                      | Ratio | Absorbance (OD) | NO Conc' (µM) |
|------|-------------------------------------------------------|-------|-----------------|---------------|
| A:B  | S. nigrum (Bungoma): S. nigrum (Kisii) methanolic extracts | 1:1   | 0.036           | < 5           |
| A:C  | S. nigrum (Bungoma): P.capensis methanolic extracts    | 1:1   | 0.038           | < 5           |
| B:C  | S. nigrum (Kisii): P.capensis methanolic extracts      | 1:1   | 0.041           | < 5           |
| D:E  | P.capensis: S. nigrum (Kisii) water extracts           | 1:1   | 0.039           | < 5           |
| D:F  | P.capensis: S. nigrum (Bungoma) water extracts         | 1:1   | 0.036           | < 5           |
| E:F  | S. nigrum (Kisii): S. nigrum (Bungoma) water extracts | 1:1   | 0.034           | < 5           |

conducted by Melaku et al. (2007) indicated that a combination of paromomycin with sodium stibogluconate was effective than when sodium stibogluconate was used alone. Similarly, a study by Ghazanfari and others (2000) demonstrated that garlic extract in combination with glucantime reduced the lesion size caused by Leishmania major more effectively than when they were alone.

Use of herbal drugs as combinations has also been in practice for centuries to treat various infectious diseases. The findings of Yousefi et al. (2009), indicated that a combination of the extracts of Alkana tincturia and Peganum harmala in a ratio of 1:1 (10:10 μg/ml) showed better in vitro effect against Leishmania major than when single extracts were used. The study conducted by Makwali et al. (2012) indicated that combination therapy with plumbaginaceae 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 of L. major-infected BALB/c mice. Another study by Ndungu et al. (2017) revealed that the water and methanolic extracts of A. secundiflora and P. capensis can be used either separately or in combination as antileishmanial therapeutic agents.

Conclusion
This study has demonstrated that combination therapy using Plumbago capensis in combination with S. nigrum resulted in complete inhibition of the growth of L. major parasites. The emergence of antimonial-resistant Leishmania strains is on the rise and natural products and other plant products that have been tested and found to possess antileishmanial activities may provide alternative treatment. On the basis of these results and considered together with existing studies that have been reported on the safe doses and side effects, combination drug therapy is a promising approach for the treatment of L. major infection.

Data availability
Dataset 1: Anti-amastigote (macrophage) assays 10.5256/f1000research.15955.d217390 (Mutoro et al. 2018b)
Dataset 2: Quantification of nitric oxide (NO) produced 10.5256/f1000research.15955.d217391 (Mutoro et al. 2018c)

Grant information
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1. The table number 1 & 2 in the results of this research article is incomplete; there is no proper labelling and description for the table. What indicating the – and + sign in the table? The other indication that needs in the second table is what representing A, B, C, D, E, F?

2. The table number 3rd shows the MIC assay, in this assay test extracts and positive control is there but what about the negative control that is only Schneider Drosophila Medium? How the concentration where fixed for extract and positive control because the deviation of concentration is large between positive control and extract (extract 2000 µg to 250 µg, and for Pento and Ampho B, it is 150 µg to 15.625 µg). The concentration of extract used here is too high.

3. The table number 3rd shows a significant difference of MIC (mg/ml) for same plant extract of S. Nigrum from the different geographical area that is Kisii and Bungoma if it is so why not for another species of plant that is P. Capensis? It is collected from only one region.

4. The table 4 indicating that the blending ratio of the plant extract inhibit the parasitic survival and there is no clear evidence to support the inhibitory effect of promastigote is really by the synergistic effect of both plant, or the quantity of active compound present in different plant extract is in different ratio?

5. In this article, the results are missing the phytochemical profile (Gas chromatographic profile and MALDI -TOF analysis) for the identification of the active compound involving the inhibitory effect of the parasite.

6. In vitro experiment, there is no clear cut idea about the drug carrier (vehicle of drug) and vehicle control.

7. The table number 6 and 7 shows the NO assay, but it is not a significant increase in between control groups (both positive and negative).

8. The manuscript lacking the immunomodulatory action of plant extract. In there in-vitro assay they should also have measured IFNg and IL 10 cytokines upon extract treatment to see the shift of Th1 and Th2 dichotomy.

9. The manuscript lacks the background knowledge about S. nigrum and P. capensis, about the medicinal property, general uses etc. and specify why they selected these two plants?
10. The result is lacking cytotoxic assay and SI index of plant extracts.

5th December: A sentence has been edited in point 6 to make the sentence clearer and in point 8, a sentence has been added regarding the *in vitro* assay. Both of which were accidentally omitted from the original report.

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

**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?**
Yes

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

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

**Are the conclusions drawn adequately supported by the results?**
Partly

*Competing Interests:* No competing interests were disclosed.

*Reviewer Expertise:* Mechanism of infection & Immunity of Visceral leishmaniasis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

Edilene O. Silva

Institute of Biological Sciences, Laboratory of Structural Biology, Federal University of Pará, Belém, Brazil

The manuscript "Efficacy of the combination of crude extracts of *Solanum nigrum* and *Plumbago capensis* on Leishmania major" by Mutoro et al submitted to the F1000Research deals with an important subject, that of bioactive compounds extraction from plants and their anti-protozoa activities.
The results reported here should be useful to the community in searching for more effective drugs to treat Leishmaniasis. The manuscript is well written but the overall approach is quite incomplete. Thus, I have a few questions/concerns about the current work, as detailed below, which should be addressed before indexing.

For any compound to be evaluated it is important that the methodology used is very clearly defined, and unfortunately this paper major lacunae is just that. In the Methodology section, the authors suggest that the leishmanicidal activity of the assayed extracts could be due to the presence of synergy between them. Could the authors provide the phytochemical profile of the assayed extracts?

Results are not properly presented and discussed. The data are presented in seven table and two figures, however not properly presented, analyzed and interpreted. Anti promastigote activity was measured using *L. amazonensis* promastigotes (10⁶ parasites/mL) and cell viability tested daily for 5 days using light microscopy. My first objection is in the use of light microscopy to measure parasite viability; it is very error prone owing to the motility of parasites, and because better semi quantitative methods are available (MTT or Alamar Blue Assay).

Later in the anti-amastigote assay, they have exposed infected macrophages to extracts, daily for 3 days post infection and cells were fixed and stained after 5 days? I am unable to understand how this approach was adopted. I think the test approach is flawed and needs to be reviewed. In addition, the authors should be performed the cytotoxicity assay.

How was in vitro dose decided? Why were high concentrations of the extracts (2000 µg/mL, 1000 µg/mL, 500 µg/mL and 250 µg/mL) used?

When data is uneven, judgment about its value is difficult. Thus, several concerns can be raised about the value of this work.

Later, in the discussion, they state that “As observed in the current study, the standard drugs were significantly more effective (P ≤ 0.05) against Leishmania promastigotes and amastigotes as compared to all the blends of the extracts. It was observed in the present study that all the blends of extracts induced little (< 1.5 µM) production of NO by peritoneal macrophages which might have played a role in the amastigote inhibition”.

So the reader ends up totally confused and demotivated to proceed to the conclusion section. Some paragraphs the authors only revise the literature. However, they do not present a discussion. This is necessary to provide the manuscript of more scientific soundness.

One way to proceed would be to request the authors to calculate do cytotoxicity test against host cell and parasites using MTT or Alamar blue assay (necessary to calculate IC₅₀). In addition, to determine phytochemical profile of the assayed extracts.

Minor mistake:

English language usage: Methods section: The word “extraction” is not used correctly, I would advise that the author use the word ‘isolation’ instead as it is more commonly used.

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

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

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?
No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: natural products against leishmaniasis

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

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