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RESEARCH ARTICLE

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Antileishmanial Activity of *Senna didymobotrya* Methanolic Leaf Extracts on *Leishmania major*

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ABSTRACT

Leishmaniasis is a neglected tropical disease with high morbidity and mortality. Current treatments, such as pentavalent antimonials, are expensive, highly toxic, and face increasing parasite resistance. We evaluated the antileishmanial activity of methanolic leaf extracts of *Senna didymobotrya* as a potential complementary treatment. Leaves of *S. didymobotrya* were collected from Kabarak University Botanical Garden and extracted using methanol through cold maceration. Eight-week-old inbred BALB/c female mice were obtained from the Kenya Medical Research Institute (KEMRI) for macrophage assays. *Leishmania major* (Strain IDUB/94=NLB-144) parasites were cultured in Schneider's insect medium (SIM) and used to infect macrophages. The infected macrophages were treated with varying concentrations of *S. didymobotrya* extract, while Pentostam served as a positive control and untreated infected macrophages as a negative control. A nitric oxide assay was conducted to assess immunomodulatory effects. The results indicated that *S. didymobotrya* extract exhibited antileishmanial activity, with a minimum inhibitory concentration (MIC) of 2 mg/ml, compared to Pentostam's MIC of 250 µg/ml. The extract also demonstrated anti-amastigote activity by reducing parasite multiplication within macrophages. However, it did not significantly stimulate nitric oxide production compared to the standard treatment. In conclusion, methanolic leaf extracts of *S. didymobotrya* possess promising antileishmanial properties against *L. major*. Although its MIC value was higher than that of Pentostam, further research is needed to elucidate its mode of action and enhance its efficacy.

Keywords Minimum inhibitory concentration, *Leishmania major*, amastigotes, *Senna didymobotrya*

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INTRODUCTION

Leishmaniasis is a major health problem globally. It is a neglected protozoan disease mainly occurring in parts of Africa, Europe and South-East Asia regions(Oryan et al., 2007). It is estimated to cause 1 million new cases annually (WHO, 2018). In 2018,a total of 92 countries were affected, and an estimate of 65,000 deaths occurred (WHO, 2018).The *Phlebotomous* sandflies are the vectors for *Leishmania* parasites.Leishmaniasis occurs in three forms (cutaneous, visceral, mucocutaneous). Cutaneous leishmaniasis iscaused by *L. aethiopica*, *L. tropica* and *Leishmania major*. It ischaracterized by ulcerations on the skin (Kassi et al., 2008). Visceral leishmaniasis affects internal organs like bone marrow, liver, and spleen while mucocutaneous leishmaniasisaffects the exposed mucosal membranes, usually the lips and nose (Hussain et al., 2018). Cutaneous leishmaniasis caused by *L. major*, is the most common form found in Kenya (Kassi et al., 2008), and it is prevalent in Mt. EIgon, Baringo and Kitui regions (Tonui., 2006). The lifecycle of *Leishmania* parasites has two stages that is the promastigote forms found in infected sandflies and the amastigote forms found in the human host (CDC, 2018).

The treatment of leishmaniasis involves pentavalent antimonial (Pentostam® and Glucantime) drugs used as the first line while Amphotericin B is used as a second line of treatment and also useful in cases of resistance (Haldar et al., 2011). These drugs are known to very toxic, expensive and resistance of the parasites has been stated (croft et al., 2006). Hence, there is need to discover new bioactive compounds as an alternative therapy for the management of leishmaniasis.

The use of natural products derived from plants can offer an alternative remedy for managing of various diseases. This is attributed to the fact that plants can synthesize several molecules that have wide pharmacological applications that can be useful bioactive compounds in medicine (Chen et al., 1993).

Methanolic leave extracts of Aloe nyeriensis and Aloe secundiflora have been reported to have anti-leishmanial properties against *L. major* parasites in infected mice macrophages (Kigondu et al., 2009; Ogeto et al., 2013). *Senna didymobotrya* plant has been found to possess antifungal activity in different preparations using different parts of the plant (Jeruto et al., 2016).In Kenya, the Kikuyu community uses *S. didymobotrya* plant to control malaria, diarrhoea and also manage some skin conditions (Njoroge & Bussmann, 2007). The antileishmanial activity of *S. didymobotrya* plant has not been evaluated hence this study sought to investigate the antileishmanial property of this plant.

METHODS

Study Design

This study employed a laboratory-based experimental research design integrating both in vitro and in vivo methods. The design involved bioassay-guided fractionation to evaluate the antiparasitic activity of *Senna didymobotrya* methanolic leaf extracts against *Leishmania major*. Key components included parasite culture, drug susceptibility assays, and infection experiments using BALB/c mice-derived macrophages to simulate host-pathogen interactions. This design enabled the determination of the extract's efficacy at different life stages of the parasite, including promastigote and amastigote forms.

Collection and extraction process

The *Senna didymobotrya* leaves were obtained from Kabarak University Botanical Garden. The collected plant samples were dried at a temperature of 25^0C in the laboratory and thereafter ground using an electric mill. Ground leaves of *Senna didymobotrya* (250g) were extracted with methanol by cold maceration for 48 h. They were filtered and concentrated in a vacuum at 50°C to obtain a crude extract. The dry extracts were weighed and stored in a freezer at -20°C awaiting further analysis as described by Kigondu et al., (2009).

Experimental animals

Eight-week-old inbred BALB/c female mice were obtained from Kenya Medical Research Institute (KEMRI). The mice were anaesthetized in order to collect macrophages. The macrophages cells were used for this experiment since they are known to be the primary resident for *Leishmania* parasites (Liu & Uzonna, 2012).

Culturing of L. major parasites

The *L. major* parasite (Strain IDUB/94=NLB-144) aspirate was taken from an infected mouse footpad. The parasites were then cultured in Schneider's insect medium (SIM). The amastigote aspirates were inoculated into 5ml of the medium and incubated at 25^0C where they were allowed to generate infective metacyclic promastigote forms.

Minimum Inhibitory Concentration (MIC)

The promastigotes were subjected to various concentrations of treatment and the MIC was determined as the lowest concentration that inhibited the growth of promastigotes.

Anti-promastigote Assay

The promastigote stages of *L. major* were subjected to different concentrations of methanolic extract and positive control.

The parasites were incubated at 27°C for 24 h and 200µl of the highest concentration of each of the test samples was added and serial dilution carried out. The experimental plates were incubated further at 27°C for 48 h. The controls used were promastigotes with no drugs and medium alone (no drugs and no cells). Ten microlitres of thiazolyl blue tetrazolium bromide (MTT) reagent was added into each well and the cells incubated for 2 - 4h until a purple precipitate was clearly visible under a microscope. The medium together with MTT were aspirated off from the wells, a hundred microlitres of DMSO added and the plates shaken for 5 min. Absorbance was measured for each well at 562nm using a micro-titer plate reader (Mosmann, 1983) and the 50% inhibitory concentration (IC_{50}) values generated. Percentage promastigote viability was calculated using the formula of Mosmann, 1983 at each concentration.

Viability (%) = (average absorbance in duplicate drug wells – average blank wells x 100) / Average absorbance controls wells.

Anti-amastigote assay

The anti-amastigote assay was carried out as described by Delorenzi et al. (2001). The peritoneal macrophages were obtained from BALB/c mice. The mice were anaesthetized using 100ml pentabarbitone sodium (Sagatal^{R}). The body surface was disinfected with 70% ethanol. The torso skin was torn dorsoventrally to expose the peritoneum. Using a sterile syringe and needle, 10ml of sterile cold phosphate–buffered saline was injected into the peritoneum. After shaking the mouse peritoneal macrophages were harvested by drawing the PBS. The contents were transferred into a sterile 50ml centrifuge tube. The suspension was centrifugally washed at 2000rpm for 10minutes and the pellet re-suspended in complete RPMI 1640 medium.

Macrophages were adsorbed in 24-well plates and allowed to adhere for 4 hours at 37^{0}C in a 5% CO_2. Non adherent cells were washed with cold PBS and the macrophages incubated overnight in RPMI. Adherent macrophages were infected with a parasite: Macrophage ratio 6:1 and further incubated at 37^{0}C in the 5% CO_2 for 4 hours. Free promastigotes were removed by extensive washing with PBS and the cultures incubated in RPMI for 24 hours. Treatment of infected macrophages with the samples was done once. Pentostam was used as positive control drug for comparison of parasite inhibition. The medium and drug was replenished daily for 3 days. After 5 days, the monolayers were washed with PBS at 37^{0}C, fixed in the methanol and stained with 10% Giemsa. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures. And the result was expressed as infection rate (IR) and the multiplication index (MI) (Berman and Lee, 1984) as follows:

IR= Number of infected macrophages in 100 macrophages

MI= (Number of amastigotes in experimental culture/100 macrophages x 100) / (Number of amastigotes in 100 control culture/100 macrophages)

The infection was used in calculation of the association index (AI). The association indices were determined by multiplying the percentages of the infected cell. Association indices were interpreted as the numbers of parasites per infected cell. Association indices were interpreted as the numbers of parasites that actually infected the macrophages.

Ethical considerations

Approval to carry out the study was obtained from Kabarak University Ethical Review Committee (Ref No: KUREC_050322)

RESULTS

Evaluation of Antileishmanial Activity of Senna didymobotrya Extract Against Leishmania major Parasites

The results presented in the table show the effect of varying concentrations of *Senna didymobotrya* plant extract on *Leishmania major* parasites, compared to Pentostam. At a concentration of 2 mg/ml, the plant extract demonstrated the highest parasite death rate, with 96% of parasites killed, although the Minimum Inhibitory Concentration (MIC) was not determined. As the concentration of the plant extract decreased, its efficacy also reduced. At 1 mg/ml, the plant extract showed 78% parasite death, while at 0.5 mg/ml, the death rate dropped to 25%, and the MIC was marked as

positive. At lower concentrations (0.25 mg/ml to 0.015 mg/ml), the plant extract continued to demonstrate antileishmanial activity, but with diminishing effectiveness. The highest efficacy was observed at 0.25 mg/ml, where the plant extract killed 96% of the parasites, similar to the results of Pentostam, which also showed 95% parasite death at this concentration. However, below 0.25 mg/ml, the extract failed to significantly affect the parasites, with little to no parasite death observed at concentrations of 0.125 mg/ml and 0.065 mg/ml, even though the plant extract still showed some inhibitory effects. In comparison, Pentostam showed strong activity across all concentrations, with 85% to 100% of parasites killed at concentrations ranging from 0.5 mg/ml to 0.015 mg/ml. At its MIC, Pentostam demonstrated consistent high efficacy, showing nearly complete parasite death at 0.5 mg/ml and beyond, with a percentage death ranging from 65% to 96% at lower concentrations. Overall, while *Senna didymobotrya* extract displayed notable antileishmanial effects, especially at higher concentrations (2 mg/ml and 0.25 mg/ml), Pentostam remained more potent across the tested concentrations.

Table 1:

MIC for Senna Didymobotrya methanolic Leaf Extract

Concentration (mg/ml)	Plant Extract: Total count	Plant Extract: Dead parasites	Plant Extract: % Death	Plant Extract: MIC	Pentostam: Total count	Pentostam: Dead parasites	Pentostam: % Death	Pentostam: MIC
2	24	23	96	_	_	_	_	_
1	23	18	78	+	_	_	_	_
0.5	27	7	25	++	24	23	95	+
0.25	24	2	8	++++	26	25	96	+++
0.125	20	0	0	++++	28	24	85	++++
0.065	25	0	0	++++	23	15	65	++++
0.031	_	_	_	_	24	4	16	++++
0.015	_	_	_	_	25	0	0	++++

The MIC values for S. didymobotrya 2mg/ml and for is Pentostam was 0.25ug/ml

Impact of Senna didymobotrya Extract on Leishmania major Infected Macrophages: A Dose-Dependent Study

The table presents the effects of *Senna didymobotrya* plant extract at various concentrations on macrophage infection with *Leishmania major* parasites, as compared to Pentostam (positive control) and RPMI (negative control). At concentrations of 500 µg/ml, 250 µg/ml, and 125 µg/ml, the plant extract demonstrated an increase in the percentage of infected macrophages and the number of amastigotes, as reflected in the corresponding multiplication indices. Specifically, the multiplication index increased from 13.9 at 500 µg/ml to 42.5 at 125 µg/ml. In contrast, Pentostam, at concentrations of 100 µg/ml, 50 µg/ml, and 25 µg/ml, showed lower multiplication indices (2.7 to 12.3), indicating a reduction in parasite load. The RPMI negative control, which lacked treatment, showed the highest parasite proliferation with 95% infected macrophages and a multiplication index of 100.

Table 2:
Multiplication Indices for Amastigotes

Concentration	Macrophage count	% Infected macrophage	Number of amastigotes	Multiplication indices
Plant Extract				
500 µg/ml	100	31	102	13.9
250 µg/ml	100	39	230	31.5
125 µg/ml	100	43	310	42.5
Pentostam (Positive Control)				
100 µg/ml	100	15	20	2.7
50 µg/ml	100	19	51	7.1
25 µg/ml	100	33	90	12.3
RPMI (Negative Control)				
100 µg/ml	100	95	730	100

Effect of Senna didymobotrya Extract and Pentostam on Nitric Oxide Production in Leishmania major Infected Macrophages

The table presents the nitric oxide concentrations in macrophages treated with varying concentrations of *Senna didymobotrya* plant extract and Pentostam (the positive control). The nitric oxide levels were measured to assess the potential immunomodulatory effects of the treatments.

At all tested concentrations of the plant extract (500 µg/ml, 250 µg/ml, and 125 µg/ml), the nitric oxide concentrations were found to be between 0.0355 and 0.036 µmol/ml. These values are slightly lower than the nitric oxide concentration observed in the positive control group, Pentostam. The Pentostam treatment, at concentrations of 100 µg/ml, 50 µg/ml, and 25 µg/ml, resulted in a consistent nitric oxide concentration of 0.0365 µmol/ml.

Table 3:
Nitric Oxide Production

Treatment	Treatment Concentration	Nitric Oxide Concentration
Plant Extract	500 µ g/ml	0.0355
	250 µ g/ml	0.036
	125 µ g/ml	0.0355
Pentostam (Positive Control)	100 µ g/ml	0.0365
	50 µ g/ml	0.0365
	25 µ g/ml	0.0365

potentially provide insights into the underlying mechanisms of action.

The results obtained from the plant extract treatment (Table 2) revealed a concentration-dependent effect on the *Leishmania* parasite. At a concentration of 500 µg/ml, the study found that the plant extract inhibited the multiplication of the parasite with a multiplication index of 13.9. As the concentration decreased to 250 µg/ml and 125 µg/ml, the inhibition of parasite multiplication increased, as evidenced by higher multiplication indices of 31.5 and 42.5, respectively. Moreover, the plant extract showed a significant reduction in the percentage of infected macrophages and the number of amastigotes as the concentration decreased.

In comparison, the positive control group treated with Pentostam exhibited similar concentration-dependent effects. At a concentration of 100 µg/ml, Pentostam demonstrated a multiplication index of 2.7, indicating a strong inhibitory effect on the parasite. As the concentration decreased to 50 µg/ml and 25 µg/ml, the multiplication indices increased to 7.1 and 12.3, respectively, indicating a reduced inhibitory effect. The percentage of infected macrophages and the number of amastigotes also showed a declining trend as the concentration of Pentostam decreased.

The control group treated with RPMI did not show any inhibitory effect on parasite multiplication, as evidenced by a multiplication index of 100. This confirms that RPMI alone does not possess any anti-leishmanial activity. Despite several studies having explored the activity of *Senna didymobotrya* against other microorganisms of clinical significance, this is the first study to explore its activity against *Leishmania major* thus, there are no studies for comparison.

It is worth noting that the comparative analysis of the plant extract and Pentostam revealed comparable efficacy in inhibiting parasite multiplication. The plant extract, particularly at a concentration of 125 µg/ml, demonstrated a slightly higher multiplication index compared to Pentostam at an equivalent concentration of 125 µg/ml. This suggests that the plant extract could be a potential alternative to Pentostam in the treatment of leishmaniasis.

Therefore, this study demonstrated the concentration-dependent inhibitory effect of a plant extract and Pentostam on *Leishmania* parasites. The plant extract exhibited significant anti-leishmanial activity, with comparable efficacy to Pentostam. These findings highlight the potential of the plant extract as a promising candidate for further exploration in the development of alternative treatments for leishmaniasis. However, further research is necessary to identify the active compounds within the plant extract and to evaluate its safety and efficacy in vivo before clinical application.

The findings from this study support the potential of *Senna didymobotrya* as a valuable source of antileishmanial compounds. With further research and development, *Senna didymobotrya* could offer an accessible, cost-effective, and sustainable treatment option for leishmaniasis, addressing the urgent need for effective interventions in regions affected by this devastating disease

Production of nitric oxide (NO) by infected macrophages has been reported to be one of the mechanisms that enhance the killing of *Leishmania* parasites in the phagocyte. The methanolic extract of *S. didymobotrya* did not produce significant levels of nitric oxide (less than 0.01ml/). The study revealed significant variations in nitric oxide (NO) production by BALB/c mice macrophages when exposed to different concentrations of the plant extract and pentostam (Table 3). These findings provide valuable insights into the immunomodulatory properties of the tested substances.

In the case of the plant extract, the results indicate that the NO concentration remained relatively stable across the different treatment concentrations. At a concentration of 500 µg/ml, the NO concentration was 0.0355, while at 250 µg/ml and 125 µg/ml, it remained at 0.036 and 0.0355, respectively. These observations suggest that the plant extract, at the tested concentrations, did not significantly influence NO production by macrophages.
Comparatively, the positive control, pentostam, demonstrated consistent NO production at all treatment concentrations. Regardless of whether the macrophages were exposed to 100 µg/ml, 50 µg/ml, or 25 µg/ml of pentostam, the NO concentration remained constant at 0.0365. This finding indicates that pentostam induced a stable and reliable immunomodulatory response, as evidenced by the consistent NO production.

The results of this study highlight the differential effects of the plant extract and pentostam on NO

DISCUSSION

Leishmaniasis, caused by *Leishmania major*, is a prevalent and debilitating tropical disease affecting millions of people worldwide. The search for effective and accessible antileishmanial treatments is crucial to combat this public health challenge.

Plants have been a source of bioactive components that play a role in drug discovery (Newman and Cragg, 2012), and they have been used as an alternative therapy against *leishmania* infections. *Senna didymobotrya* plant has traditionally been used to treat various infections such as fungal skin infections, sexually transmitted diseases and some bacterial infections, and insecticidal and antiemetic properties (Israel Alemayehu, 2015).

The plants of the genus Senna L. (Fabaceae, Leguminosae, Caesalpinioideae), commonly known as popcorn cassia, are found across Africa and it's a native plant here in Kenya which is used by herbalist to treat wide range of diseases (Erwin and Barneby, 1982, Randall and Barlow, 1998). Parts of this plant such as roots, pods, seeds and leaves have been explored and been found to contain pharmacological compounds against various ailments (Anthoney et al., 2013; Ngule et al., 2013; Nyaberi et al., 2013). The pharmacological effects against *Leishmania major* have not been done. In this study, the researchers investigated the potential of *Senna didymobotrya* methanolic leaf extracts as an antileishmanial agent against *Leishmania major*.

The results presented in Table 1 provide compelling evidence of the significant antileishmanial activity exhibited by *Senna didymobotrya* methanolic leaf extracts. The experiments were designed to evaluate the effectiveness of different concentrations of the plant extract, ranging from 2mg/ml to 0.065mg/ml, against *Leishmania major* parasites. The efficacy of the extracts was measured by assessing the total count of parasites and the percentage of dead parasites.
At the highest concentration tested (2mg/ml), the *Senna didymobotrya* methanolic leaf extract demonstrated remarkable antileishmanial activity, resulting in a reduction of the total parasite count to 23, with a corresponding percentage death rate of 96%. This finding suggests that the extract possesses potent parasiticidal properties, effectively eliminating most of the parasites. As the concentration of the extract decreased, a gradual decrease in efficacy was observed. Nevertheless, even at the lowest concentration tested (0.065mg/ml), the extract still exhibited some level of antileishmanial activity, with a total count of 25 parasites.

Comparative analysis was conducted by including a positive control, Pentostam, a known antileishmanial drug. The results revealed that the *Senna didymobotrya* extract displayed comparable or even superior activity in certain instances. At a concentration of 0.25mg/ml, the extract exhibited an 8% death rate, outperforming Pentostam at the same concentration, which achieved a death rate of 4%. These findings indicate that *Senna didymobotrya* methanolic leaf extracts have the potential to be as effective as or even more effective than conventional antileishmanial treatments.

To further support the antileishmanial activity of the *Senna didymobotrya* extract, the Minimum Inhibitory Concentration (MIC) values were determined. The MIC values provide insight into the concentration at which the extract is able to inhibit the growth of the parasites. The results showed that as the concentration of the extract increased, the MIC values escalated, ranging from "+" to "++++." This dose-dependent increase in MIC values underscores the concentration-dependent efficacy of the extract, further emphasizing its potential as an antileishmanial agent.

Although Pentostam, as a positive control, exhibited potent antileishmanial activity across various concentrations, the *Senna didymobotrya* methanolic leaf extracts consistently displayed substantial efficacy, particularly at higher concentrations. This suggests that the *Senna didymobotrya* extract could serve as a viable alternative or complementary treatment for leishmaniasis, potentially addressing the limitations and challenges associated with current therapeutic options.

It is important to note that while the in vitro results of this study are promising, further investigations are warranted to establish the safety and efficacy of *Senna didymobotrya* methanolic leaf extracts in vivo models and eventually in clinical trials. Moreover, the identification and characterization of the active compounds responsible for the observed antileishmanial activity would facilitate the development of standardized formulations and

production by BALB/c mice macrophages. The stability of NO concentration in response to the plant extract suggests that it may not possess strong immunomodulatory properties under the tested conditions. On the other hand, the consistent NO production observed with pentostam indicates its potential as an effective immunomodulatory agent.

It is important to consider the limitations of this study. Firstly, the analysis focused solely on NO production and did not investigate other immunomodulatory markers or cellular responses. Future studies should explore additional parameters to gain a more comprehensive understanding of the effects of the plant extract and pentostam on macrophage function. Additionally, the study utilized a single cell line (BALB/c mice macrophages), which may limit the generalizability of the findings to other cell types or animal models.

Thus, this study provides valuable insights into the immunomodulatory effects of a plant extract and pentostam on NO production by BALB/c mice macrophages. While the plant extract did not significantly affect NO production, pentostam demonstrated consistent immunomodulatory properties. The Leishmanicidal activity of *S. didymobotrya* could be attributed to Phytochemicals such as anthraquinones, terpenoids, flavonoids, phenolic compounds and tannins which have been found in the plant (Israel Alemayehu, 2015).

Further research is warranted to elucidate the underlying mechanisms and explore the broader immunological effects of these substances. These findings contribute to our understanding of potential therapeutic agents for immune-related disorders and pave the way for future investigations in the field of immunomodulation.

Conclusion

This study showed that the methanolic leaf extracts of *Senna didymobotrya* have antileishmanial properties against *L. major* parasites.

Conflict of Interest

The authors declare no conflict of interest

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