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In vitro activity of aqueous and methanol extracts of *Callistemon citrinus* (Family Myrtaceae) against *Leishmania major*.

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SUMMARY

***Leishmania major* is a protozoan parasite that causes cutaneous leishmaniasis and the standard drugs are expensive and toxic. Cheaper and safer natural drugs are therefore needed. In this study, the *in vitro* efficacy of crude extracts of *Callistemon citrinus* were tested against *L. major.* Controls were anti leishmanial drugs pentostam and liposomal amphotericin B. The minimum inhibitory concentrations of *C. citrinus* crude aqueous and methanolic extracts were 5mg/ml and 1mg/ml respectively compared to 12.5μg/ml and 6.25μg/ml for pentostam and liposomal amphotericin B respectively. The IC_{50} for *C. citrinus* extracts against promastigotes ranged from 297.75 to 572.69μg/ml compared to 0.26 and 0.82μg/ml for pentostam and liposomal amphotericin B. The IC_{50} for *C. citrinus* extracts against vero cells ranged from 467μg/ml to 1314.65μg/ml. The promastigotes' viability after treatment with aqueous and methanolic extracts was 69.58% and 75.74% respectively. At 125μg/ml, the aqueous and methanolic *C. citrinus* extracts had *in vitro* amastigotes' infection rates (IRs) of 77.0±2.50 % and 77.5±3.50% respectively. The multiplication indices (MIs) and IRs of amastigotes treated with *C. citrinus* crude aqueous extracts and those treated with crude methanolic extracts differed insignificantly ($P > 0.05$). *C. citrinus* methanolic extracts stimulated production of about 20μM nitric oxide in BALB/c mice peritoneal macrophages suggesting immuno-modulatory role of the extracts. The crude aqueous and methanolic extracts of *C. citrinus* were therefore concluded to be relatively less toxic and possessed *in vitro* anti-leishmanial activity against *L. major* promastigotes and amastigotes.**

Key Words: *Callistemon citrinus*, crude extracts, *Leishmania major*, cutaneous leishmaniasis, antileishmanial activity, vero cells

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Introduction

Leishmaniases are caused by flagellate protozoan parasites of the genus *Leishmania* which are transmitted by the blood sucking phlebotomine sand flies. Approximately 12 million people in 88 countries are infected with leishmaniases and 2 million new cases occur each year [1]. Cutaneous leishmaniasis (CL) is the most common form of *Leishmania* infections with an estimated incidence range of 0.7 to 1.2 million cases each year [2]. CL often causes skin lesions, pain, stigmatization, and psychological emotional problems [3]. In the Old World, CL is caused by *Leishmania major* among others [4]. *L. major* has been reported in Baringo County, in Kenya [5].

The drugs for leishmaniases have been pentavalent antimonials, mainly sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) [6]. Despite their toxicity, antimonials are still the first line leishmaniases drugs in most areas [7]. Amphotericin B and pentamidine are alternative leishmaniases drugs which are of greater toxicity. Liposomal amphotericin B is however less toxic and more effective but expensive. Leishmaniases drugs are associated with a prolonged healing time and serious side effects [8], and majority of the drugs are administered intravenously which calls for hospitalization of the patient [9]. Quite often, relapse of the disease after an initial chemotherapy treatment occurs [10] and drug resistance has been reported in endemic areas [11]. Therefore, cheaper, more effective and less toxic drugs are urgently needed.

Herbal or natural products are increasingly becoming important in search of drugs because they are safer in contrast to the synthetics [12]. *Callistemon citrinus* (Family Mrytaceae) is an ornamental evergreen tree in Kenya commonly referred to as the red bottle brush. Plants of the genus *Callistemon* possess significant activities as antistaphylococcal, nematocidal, larvicidal, pupicidal, antioxidants, antithrombotic and antifungal [13] [14]. In folk medicine, the genus *Callistemon* is known as anti-cough, anti-bronchitis, and insecticidal agent [15] in addition to being a treatment of gastro-enteritis, diarrhea and skin infections. Research on anti-leishmanial activity of *C. citrinus* extracts has not been carried out in Kenya. It is against this background that the present study was designed to investigate on the *in vitro* activity of *C. citrinus* crude aqueous and methanolic extracts against *L. major*.

Materials and Methods

Plant materials

The study plant *C. citrinus* samples were harvested from selected homesteads in Nakuru County, Kenya. They were positively identified as *C. citrinus* (Curtis) Skeels at University of Nairobi Herbarium, Department of Botany at Chiromo campus. The flowers were picked and taken to Kenya Medical Research Institute (KEMRI) *Leishmania* laboratory in Nairobi where they were dried at room temperature until they became brittle and attained a constant weight. The dried flowers were then transferred to the Center for Traditional Medicine & Drug Research (CTMDR) at KEMRI, where they were ground and extracted using water and analytical grade methanol.

Plant Extracts

The aqueous and methanolic extracts were prepared as described by Delahaye *et al.* [16] and Cock [17] respectively. Briefly, 50 g of the ground dried flower material in 600 ml of distilled water was placed in a water bath at 70°C for 1.5 hours. The mixture was

filtered using Whatman No 1 filter papers and the filtrate freeze dried. Similarly, 100g of ground plant material was soaked in 500 ml of analytical grade methanol for 72 hours at room temperature with gentle shaking, then filtered and concentrated using a rotary evaporator to obtain the methanolic extracts. The dry extracts were weighed and stored at 4°C until required for the bioassays. The aqueous *C. citrinus* extracts were coded as extract B while the methanolic extract was coded as extract G. The yields for aqueous extracts B was 8.79g (17.58%) while that of methanolic extracts G was 9.24g (9.24%).

Leishmania parasites

Leishmania major strain (IDUB/KE/94=NLB-144) was used in this study. The parasite was originally isolated from female *Phlebotomus duboscqi* sand fly that had been collected near Marigat, Baringo County, Kenya [18]. This strain was acquired from Institute of Primate Research, Kenya (IPR, Kenya) where it had been maintained by cryopreservation in liquid nitrogen. The parasites were grown to stationary phase at 25°C in Schneider's insect medium supplemented with 20% heat inactivated fetal bovine serum (FBS-HYCLONE® USA), 100 U/ml penicillin and 500μg/ml streptomycin [19], and 250μg/ml 5-fluorocytosine arabinoside [20]. The stationary-phase metacyclic stage promastigotes were then harvested by centrifugation at 1500 rpm for 15 minutes at 4°C. The metacyclic promastigotes were then used for the *in vitro* assays.

Vero cells culture

The Vero cells were obtained from Kenya Medical Research Institute (KEMRI), Nairobi, from the Center for Viral Research (CVR) where they had been cryopreserved in liquid nitrogen to ensure the best quality. Briefly, the vero cells (100 μl) were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS (FBS-HYCLONE® USA), 100U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with an atmosphere of 5 % CO_2 in air at 37°C. The vero cells were plated at a concentration of 3×10^5 cells per ml of the culture medium in petri dishes and allowed to grow for 24 hours until a well spread monolayer was formed. The vero cells monolayer was then used for cytotoxicity studies.

Experimental animals

Eight week old inbred BALB/c mice of the same sex were used for *in vitro* macrophage assay for the plant extracts. The inbred BALB/c mice were obtained from International Livestock Research Institute (ILRI), Kenya. They were then housed at the KEMRI animal house at 23°C to 25°C and were fed on standard commercial diet in the form of mice pencils and were given tap water *ad libitum*. The mice were handled in accordance with the regulations that have been set by KEMRI's Animal Care and Use Committee (ACUC).

Evaluation of minimum inhibitory concentration (MIC)

The MIC was determined as described by Wabwoba *et al*. [21]. Promastigotes at a concentration of 1×10^6 metacyclic promastigotes per ml of culture medium were exposed to several concentrations of the test plant extracts. The lowest concentration of the test plant extracts that inhibited promastigotes growth was taken to be the MIC.

Anti-promastigote assay

Anti promastigote activity of the test extracts was evaluated as described by Wabwoba *et al*. [21]. The metacyclic promastigotes at a concentration of $1x10^6$ promastigotes per ml of the medium were incubated in 24-well plates in the presence of different

concentrations of the plant extracts for five days at 25°C. The aliquots of parasites were then transferred into 96-well micro-titer plates (Corning, NY, USA), incubated at 27°C in 5% CO_2 for 24 hours and 200µl of the test extracts B and G samples were added at concentrations ranging from 5 mg/ml to 0.5 mg/ml. The plates were then incubated further at 27°C for 48 hours. The control wells contained culture alone. 10µl of 2, 5-diphenyltetrazolium bromide (MTT) reagent was added into all the wells and incubated for 4 hours after which the medium together with MTT were aspirated off the wells. In each well, 100µl of Dimethyl sulfoxide (DMSO) was added and the plates shaken for 5 minutes. Absorbance was measured for each well at 562 nm using a micro-titer reader [22]. The absorbance readings were used to generate the 50% inhibitory concentration (IC_{50}) values for extracts B and G. Percentage promastigotes' viability (%) was determined as described by Mossman [22] by using optical absorbance of treated and untreated samples and blank wells in the formula: Viable promastigotes (%) = $(AT - AB) / (AC) \times 100$, where *AT* was the absorbance of treated samples and *AB* was the absorbance of the blank wells and *AC* was the absorbance of the control wells.

Anti-amastigote assay

The anti-amastigote assay was carried out as described by Delorenzi *et al.* [23]. Briefly, peritoneal macrophages were obtained from clean BALB/c mice. 10 ml of sterile cold phosphate-buffered saline (PBS) was injected into the peritoneum of anaesthetized BALB/c mice whose body surface had been disinfected with 70% ethanol. The PBS containing the macrophages was washed through centrifugation at 2,000 rpm for 10 minutes and the macrophages were adsorbed in sterile 24-well plates for 4 hours at 37°C in 5% CO_2. Adherent macrophages were infected with promastigotes and further incubated at 37°C in 5% CO_2 for 4 hours and then washed with sterile PBS to remove the free promastigotes. This was followed by further incubation of the infected macrophages for 24 hours in RPMI 1640 culture medium. The infected macrophages were then treated once with extracts B and G. Pentostam and liposomal amphotericin B were used as positive control drugs. The medium, test extracts and control drugs were replenished daily for 3 days. After 5 days, the macrophages were washed with 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 results was expressed as infection rate (IR) and multiplication index (MI) as described by Berman & Lee [24].

Determination of nitric oxide production

Measurement of nitric oxide (NO) production was carried out as described by Gamboa-Leon *et al.* [25]. Briefly, BALB/c peritoneal macrophages were seeded at 1×10^5 cells per well in 96-well culture plates (Corning, NY, USA) and allowed to adhere at 37°C in 5% CO_2-humidified atmosphere. Two hours later, the peritoneal macrophages were incubated in RPMI-1640 with 10% FBS, and incubated further for 48 hours in presence of aqueous extracts B or methanolic extracts G or RPMI-1640 medium. At least a 100 µl of supernatants were collected after the incubation of the peritoneal macrophages with the test extracts and stored for further tests. NO released was measured using the Greiss reaction for nitrites (NO_2^-) as described by Hollzmuller *et al.* [26]. A nitrite standard reference curve was first prepared by dispensing 100µl of 100µM sodium nitrite solution into the first 3 wells in row A of a 96- well micro titer plate, followed by a six series two fold

dilutions (50μl/well) down the plate (columns 1, 2, & 3) . The standard curve generated corresponded to the concentrations 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 μM. Secondly, 50 μl of the supernatants initially obtained from the macrophage cultures treated with aqueous extracts B and methanolic extracts G, were separately added into the wells in duplicate, starting from column 4 of the micro titer plate. 50 μl of Greiss reagent A (1% sulphanilamide in 5% phosphoric acid) was dispensed to all the experimental samples and into the wells containing sodium nitrite solution. Following an incubation of 5 minutes, 50 μl of Greiss reagent B (0.1 % N- [1-naphthyl] ethylenediamine dihydrochloride (NED) in water) was dispensed into all the wells and incubated for 5 minutes before measuring the absorbance at 520-550 nm in a micro titer plate reader. The average absorbance values for the standard nitrite, aqueous extracts B, methanolic extracts G and RPMI-1640 medium were calculated and the trend indicated in a line graph.

Determination of cytotoxicity of the extracts on vero cells.

The assay was carried out as described by Wabwoba *et al.* [21]. The vero cells were harvested by trypsinization, and pooled in 50 ml centrifuge tubes from where a 100μl of the cell suspension (1×10^6 cells/ml) were obtained and put into 2 wells of rows A-H in a 96-well flat bottomed micro-titer plate. The vero cells were incubated in Minimum Essential Medium (MEM) supplemented with 10% FBS, penicillin 100 IU/ml and streptomycin 100 μg/ml at 37°C in humidified 5% CO_2 for 24 hours to attach, after which the medium was gently aspirated off and 150 μl of the highest concentration (1000μg/ml) of aqueous extracts B and methanolic extracts G was added and serially diluted by a factor of two up to a concentration of 15.63μg/ml. The vero cells exposed to extracts were further incubated at 37°C for 48 hours in a humidified 5% CO_2 air atmosphere. The controls comprised of wells with vero cells treated with serially diluted pentostam and liposomal amphotericin B drugs. The blank wells with medium alone were included to assist in determining the viabilities of the cells as described by Mossman [22]. MTT reagent (10μl) was added into each well and then incubated for 2 to 4 hours until purple precipitate (formazan) was visible under the microscope. The medium and MTT reagent were gently aspirated off, after which 100 μl of 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 micro-titer plate reader at wavelength of 570 nm [27]. Cell viability was also calculated at each concentration as described by Mossman [22]. The IC_{50} values of the extracts were determined using the Chemosen software program.

2.11 Data analysis

Data was analysed using SPSS version 17.0 for windows at 5% level of significance. One way ANOVA (F test) was used to compare promastigotes and vero cells viability (%) after exposure to different concentrations of extracts B, G and the standard drugs. Other variables compared using F test were infection rates (IRs) and multiplication indices (MIs) of amastigotes in peritoneal macrophages, and also nitric oxide production in peritoneal macrophages. A situation where homogeneity test of variance (Levene's test) was significant, robust tests of equality of means that included Brown-forsythe and Welch tests were carried out as alternative versions of the F-statistics. Multiple comparisons of the individual treatments were done

using both Tukey HSD and Games-Howell *post hoc* tests.

Results

The MICs, IC_{50} values of the test extracts and viabilities (%) of promastigotes.

The MIC of *C. citrinus* crude aqueous extracts was 5000μg/ml while that of the crude methanolic extract was 1000μg/ml. The standard *Leishmania* drugs pentostam and liposomal amphotericin B had MICs of 12.5μg/ml and 6.25μg/ml respectively (Table 1). The IC_{50} values for the crude aqueous and methanolic *C. citrinus* extracts were 297.75 and 572.69 μg/ml respectively while the viabilities (%) of the promastigotes were 75.74% and 69.58% respectively (Table 2). The promastigote reduction (%) associated with the crude aqueous and methanolic extracts with reference to Schneider's Insect medium (SIM) was 7.24% and 14.78% respectively as compared to 77.45% and 85.03% for pentostam and liposomal amphotericin B respectively.

Table 1: Survival of the *L. major* promastigotes in varying concentrations of the plant extracts as observed under a light microscope.

		Concentrations of the extracts (μg/ml)					
Test extracts & Controls	Code	5000	4000	3000	2000	1000	500
Test extracts							
Aqueous *C. citrinus*	B	–[a]	+	++	+++	++++	++++
Methanolic *C. citrinus*	G	–	–	–	–	–	+
Positive Controls:		Concentrations of the standard drugs (μg/ml)					
		100	50	25	12.5	6.25	3.125
Pentostam	Pent	–	–	–	–	+	++
Liposomal amphotericin B	AmpB	–	–	–	–	–	+
Negative control							
Schneider's Insect Medium	SIM	++++[b]	++++	++++	++++	++++	++++

–[a]: shows that there were no live promastigotes observed; ++++[b]: four positives represent maximum survival (high density) of the promastigotes;

Table 2: The MICs, IC_{50} values and viabilities (%) for the *L. major* promastigotes after exposure to varying concentrations of aqueous and methanolic bottle brush extracts.

Test extracts & controls	MIC Code	(*m*g/ml)	IC_{50} (*μ*g/ml)	$-\log_{10}IC_{50}$ (pIC_{50} scale)	Viability (%) (5– 0.5mg/ml)[a]
Test extracts					
Aqueous *C. citrinus*	B	5	297.75	−2.47	75.74
Methanolic *C. citrinus*	G	1	572.69	−2.76	69.58
Positive controls[b]					
Pentostam	Pent	0.0125	0.26	0.59	18.41
Liposomal amphotericin B	AmpB	0.00625	0.82	0.09	12.22
Negative control					
Schneider's Insect Medium	SIM	–	–	–	81.65

[a] = Concentration of the extracts ranged between 5000 to 500 *μ*g/ml (5 to 0.5 mg/ml); [b] = the initial concentration of the control drugs was 100 *μ*g/ml (0.1mg/ml) followed by serial dilution by a factor of 2.

Infection rate and multiplication index of the amastigotes.

At a concentration of 125*μ*g/ml, the *C. citrinus* aqueous extract B and methanolic extract G were associated with amastigotes infection rates (IRs) of 77.0 ± 2.50 % and 77.5 ± 3.50 % respectively (Table 3) implying that they inhibited survival of amastigotes in BALB/c peritoneal macrophages by 9.05% and 8.47% respectively in reference to control RPMI-1640 medium whose IR was 84.67 ± 2.96 %. On the contrary, the control leishmaniases drugs, liposomal amphotericin B and pentostam at concentration of 50*μ*g/ml inhibited the *in vitro* survival of the amastigotes in macrophages more as indicated by their low IRs of 5.5 ± 0.50% and 11.0 ± 3.00% respectively. The trend was similar for multiplication indices (MIs) for both the test extracts and the control drugs (Table 3). Liposomal amphotericin B *in vitro* activity against amastigotes was therefore more efficacious than that of pentostam at the same concentration. Higher concentrations of both the test extracts and the control drugs resulted to low IRs and MIs of *L. major* in BALB/c mice peritoneal macrophages *in vitro.* Their efficacy was therefore dose-dependent. In a concentration ranging from 15.63 *μ*g/ml and 125 *μ*g/ml, the IRs and MIs of aqueous extract B and those of methanolic extract G differed insignificantly ($P > 0.05$). Robust test of equality of means showed that there was significant difference ($P < 0.05$) between the IRs and MIs of both aqueous extract B and methanolic extract G and those of the drugs pentostam and liposomal amphotericin B.

Table 3: *In vitro* infection rates (IR) and multiplication indices (MI) of *L. major* amastigotes upon treatment with standard drugs and *C. citrinus* aqueous extract B and methanolic extract G in duplicate cultures.

Drug	Conc (μg/ml)	Macrophages Number	IR (%)	Amastigotes/100cells	MI (%)
Aqueous	125.00	100	77.0±2.50[a]	293.5 ± 12.50	66.28
extract B	62.50	100	80.5±3.00	309.0 ± 11.50	69.91
	31.25	100	86.0± 1.50	363.0 ± 6.50	82.13
	15.63	100	85.0±2.00	350.5 ± 10.00	79.19
Methanolic	125.00	100	77.5±3.50	269.5 ± 23.50	60.97
extract G	62.50	100	81.0±3.00	294.5 ± 14.50	66.62
	31.25	100	83.0±3.00	307.5 ± 15.50	69.45
	15.63	100	87.5±1.50	331.5 ± 18.50	75.00
Pentostam	50.00	100	11.0±3.00	50.0 ± 1.00	11.31
	25.00	100	36.5±1.50	133.0 ±10.00	30.09
	12.50	100	66.0±1.00	198.5 ±11.50	44.90
	6.25	100	79.0±2.00	318.5 ±11.50	71.94
Lipo Amph B	50.00	100	5.5±0.50	40.0 ± 2.00	9.05
	25.00	100	9.5±0.50	46.5 ± 2.50	10.52
	12.50	100	14.5±4.50	52.0 ± 2.00	11.76
	6.25	100	22.0±1.00	71.5 ± 9.50	16.18
RPMI-1640[b]	1st	100	89	451	n/a
(Medium)	2nd	100	79	403	n/a
	3rd	100	86	472	n/a
	Average	100	84.67±2.96	442 ± 20.42	100.00

[a] refers to plus or minus standard errors (SE); RPMI[a] refers to negative control in which the amastigotes multiplied maximally.

Effect of C. citrinus extracts on nitric oxide production by macrophages

Since the breakdown of NO occurs in the macrophages to release nitrite (NO_2^-) as one of the products, NO produced was estimated by quantifying the NO_2^- content. The representative nitrite standard reference curve which was in RPMI-1640 medium was prepared as described in the Griess Reagent System instructions (Promega® USA). The aqueous extracts B at concentrations that ranged between 3.125 and 100 μg/ml triggered production of negligible amount of NO (< 2μM) and this compared closely with RPMI-1640 medium which also stimulated insignificant NO production (Figure 1). However, the methanolic G extracts stimulated production of about 20μM of NO at concentrations of 6.25μg/ml (Figure 2).

Figure 1: Amount of nitric oxide (µM) produced by macrophages treated with different concentrations of *C. citrinus* aqueous extract B (100μg/ml serially diluted to 3.125μg/ml by a factor of 2).

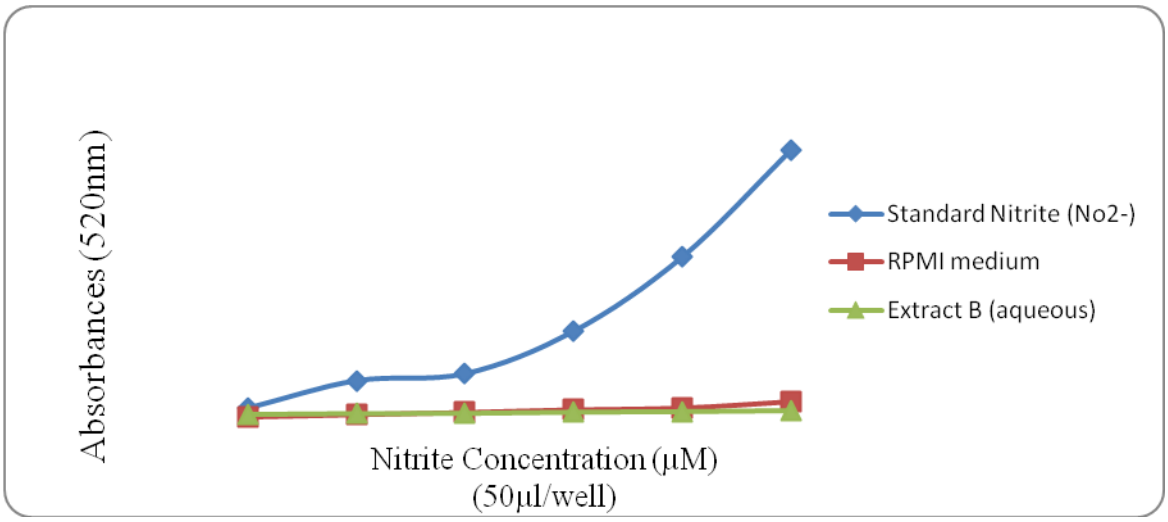
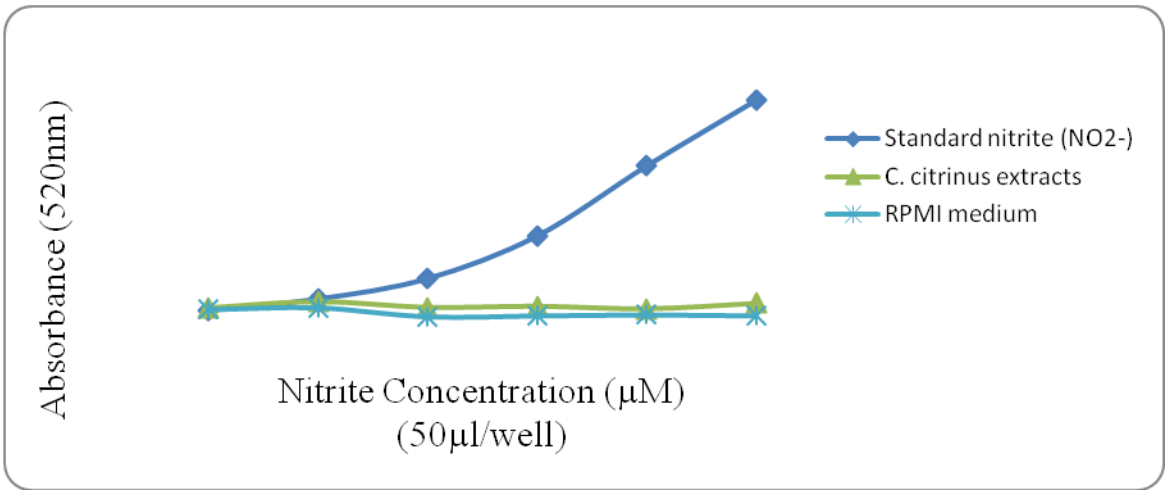


Figure 2: Amount of nitric oxide (µM) produced by macrophages treated with different concentrations of *C. citrinus* methanolic extract G (100μg/ml serially diluted to 3.125μg/ml by a factor of 2).



Cytotoxicity of the aqueous and methanolic C. citrinus extracts on vero cells

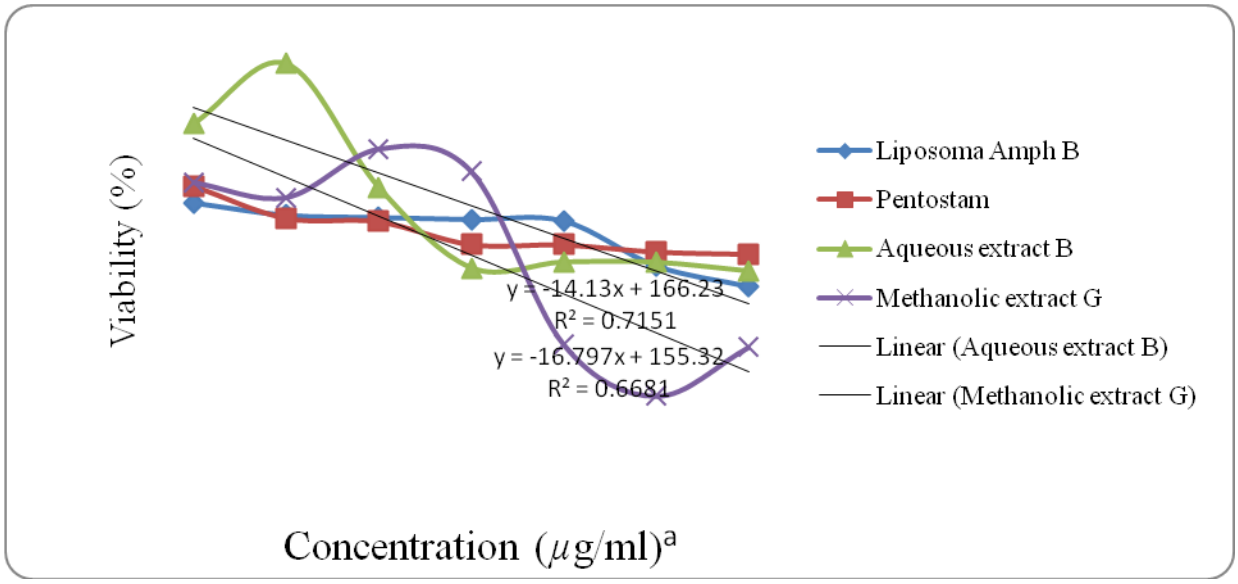
The *C. citrinus* aqueous extract B had an IC_{50} of $467\mu g/ml$ while the methanolic extract G had IC_{50} values of $1314.65\mu g/ml$ respectively. Similarly, the IC_{50} values of the control drugs, pentostam and liposomal amphotericin B were $108.58\mu g/ml$ and $60.95\mu g/ml$ respectively. At concentration ranging from $250\mu g/ml$ to $1000\ \mu g/ml$, aqueous extract B and methanolic extract G corresponded to vero cells' viabilities ranging from 85%, and slightly less than 50% respectively (Table 4). However, the line graph showed that methanolic extract G at a concentration of $250\mu g/ml$ corresponded to 50% viability of vero cells (Figure 3). In comparison, the vero cells viability after exposure to $100\mu g/ml$ of pentostam and liposomal amphotericin B were 74% and 88% respectively (Table 4). Therefore viability of vero cells upon exposure to *C. citrinus* aqueous extract B at concentration of $125\mu g/ml$ compared closely with that of pentostam at concentration of $50\mu g/ml$ (Table 4). Both aqueous and methanolic *C. citrinus* extracts showed a dose-dependent cytotoxic effect on vero cells as indicated by the trend lines (Figure 3).

Table 4: Viability (%) of vero cells after *in vitro* exposure to specific concentrations ($\mu g/ml$) of *C. citrinus* extracts.

Plant extracts/ Control drugs	Code	Vero cells viability (%) at different concentrations ($\mu g/ml$) 1000	500	250	125	62.50	31.25	15.63
Aqueous *C. citrinus*	B	81.32	85.08	85.21	82.70	117.49	171.02	145.15
Methanolic *C. citrinus*	G	48.37	27.29	49.70	124.49	134.14	113.25	119.69
Vero cells viability (%) at different concentrations ($\mu g/ml$)								
Positive controls		100	50	25	12.5	6.25	3.125	1.563
Pentostam	Pent	74.83	83.74	103.17	103.79	104.65	105.65	110.92
Lip amphotericin B	AmpB	88.83	89.82	92.86	92.99	102.79	104.28	118.00
Negative control[a]								
Minimum essential medium	MEM	Vero cells viability (%) = 92.86 ± SD 0.16						

[a] The % viability in the negative control was the average for viabilities of vero cells in MEM in microtiter plates A and B.

Figure 3: The trend of vero cells' viability as the concentrations of the *C. citrinus* aqueous and methanolic extracts and that of control drugs (pentostam and amphotericin B) increased.



Note: [a] = the concentration for the control drugs ranged between 100μg/ml and 1.563μg/ml, with a dilution factor of 2.

Discussion

From literature, little research has been carried out on anti-protozoal properties of *C. citrinus* extracts. Majority of the investigations on medicinal properties of *C. citrinus* extracts have been focusing on antibacterial potential. The *C. citrinus* flowers extracts have been reported to be potent inhibitors of bacterial growth [17]. It was observed from the present study that crude *C. citrinus* aqueous and methanolic flowers extracts were also effective against *L. major* promastigotes *in vitro* at concentrations ranging from 1 to 5 mg/ml. The crude *C. citrinus* aqueous and methanolic crude flowers extracts were less toxic (IC_{50} of 297.75 and 572.69 μg/ml respectively) and were associated with *L. major* promastigotes' viabilities of 75.74% and 69.58% respectively. The standard *Leishmania* drugs pentostam (IC_{50} value of 0.26μg/ml) and liposomal amphotericin B (IC_{50} value of 0.82μg/ml) were associated with *L. major* promastigotes viability of 18.41% and 12.22% respectively. Although the standard drugs are effective against promastigotes, they are highly toxic, and furthermore they require a protracted administration in patients [28]. The essential oils from *C. citrinus* when tested against some species of bacteria have been reported to have low MIC values that range between 0.31 and 2.5 mg/ml [29]. In the present study, the crude methanolic flowers extracts of *C. citrinus* had MIC of 1.0 mg/ml against *L. major* promostigotes and this was in agreement to the range of MICs reported by Oyedeji *et al.* [29]. Similarly, both aqueous and methanolic extracts of *Callistemon viminalis* were found to exhibit MICs of 1.6 mg/ml against *Staphylococcus aureus in vitro* [16] which was also in the range of 1.0 mg/ml obtained for crude methanolic *C. citrinus* flowers extracts in the present study. Seyydnejad *et al.* [30] observed that the MIC of *C. citrinus* methanolic and ethanolic leaves

extracts against several species of bacteria ranged between 5.00 and 40.00 mg/ml which again agreed with the MIC of 5 mg/ml for aqueous *C. citrinus* extracts against *L. major* observed in the present study. In some other studies however, ethanolic extracts of *C. citrinus* leaves have been reported to show low MIC values ranging from 0.02 to 0.31 mg/ml against several species of bacteria [31].

The protozoan parasite *L. major* infects mononuclear phagocytes, and therefore the control of infection depends on adequate activation of the infected macrophages to kill parasites and inhibit their replication [32]. In this regard, the efficacy of *C. citrinus* aqueous and methanolic extracts in inhibiting the replication of amastigotes in infected BALB/c peritoneal macrophages was quantified by calculating their infection rate (IR) and multiplication indices (MI). Based on the IRs, the *C. citrinus* aqueous crude flowers extracts B had a parasite inhibition rate of 9.05% while the methanolic crude flowers extracts G had 8.47 %, both at a concentration of 125μg/ml. In comparison, pentostam and liposomal amphotericin B had parasite inhibition rate of 22.05% and 82.87% respectively at a concentration of 12.50μg/ml. There was a significant difference between the efficacy of the test crude extracts and that of the *Leishmania* drugs ($P < 0.05$). This observation indicated that *C. citrinus* extracts which are known for their antibacterial and anti-fungal potential [16], also possessed anti-leishmanial activity. According to Oyedeji *et al.* [29], *C. citrinus* and *C. viminalis* leaves are known to be rich in 1, 8-cineole (eucalyptol) and alpha pinene essential oils. Both alpha pinene and 1, 8-cineole are non polar terpenes [16]. Since linalool is a monoterpene that has a strong anti-leishmanial activity [33], there is a high possibility that eucalyptol and alpha pinene may also possess anti-leishmanial activity.

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 [25]. In this study, it was observed that crude methanolic extracts of *C. citrinus*, stimulated production of 20μM of NO at concentrations of 6.25μg/ml (Figure 2). It was also observed in the present study that *C. citrinus* crude flowers extracts had inhibitory effects against amastigotes in peritoneal macrophages *in vitro.* The NO produced might have played a role in the amastigote inhibition. Other studies have shown that treatment of pre-infected murine macrophages with 15 ng/ml of linolool-rich essential oil (monoterpene) not only reduced the interactions between the macrophages and the parasite by 50%, but it also increased NO production [33]. Previous studies have also reported that essential oils in the extracts of *C. citrinus* and *C. viminalis* contain linolool components [29]. This would therefore suggest that the NO production associated with *C. citrinus* methanolic flower extracts in the present study possibly was contributed by their linolool fractions. Aqueous *C. citrinus* crude extracts were however observed to induce negligible NO production by peritoneal murine macrophages although the extracts had inhibitory effects against the amastigotes *in vitro*. This may require further investigations.

The *C. citrinus* aqueous flower extracts (IC_{50} = 467μg/ml) and methanol flower extracts (IC_{50} = 1314.65μg/ml) were less toxic to vero cells when compared to pentostam (IC_{50} = 108.58μg/ml) and liposomal amphotericin B (IC_{50} = 60.95μg/ml). Many drugs used for leishmaniasis treatment are highly toxic

[34], and this is an observation which the present study concurs with. Both the *C. citrinus* aqueous and methanolic flowers extracts showed a dose-dependent cytotoxic effect in which a high dose was more toxic and vice versa as illustrated by the linear trend lines in Figure 3. Methanolic extracts of *C. citrinus* have previously been reported to be toxic towards the aquatic brine shrimp, *Artemia franciscana nauplii* [35]. Similarly, Firoz *et al*. [36] demonstrated that ethanol extracts of *Callistemon lanceolatus* (synonym: *C. citrinus*) results to high mortality in albino wistar rats of body weight 150-250 g at a dose of 3g/kg. Extracts of *C. citrinus* are rich in essential oils that include 1,8-cineole and alpha pinene [29] and according to Koul *et al*. [37], the terpenoid constituents of essential oils can be moderately toxic to mammals. 1,8-cineole has previously been demonstrated to have a toxicity level of LD_{50} 2480 mg/kg when administered orally in experimental rats [37]. Probably the moderate *in vitro* cytotoxicity of *C. citrinus* extracts to vero cells observed in the present study was partly due its essentials oils components.

Conclusion

From this study, it can be concluded that the test crude aqueous and methanol flowers extracts of *C. citrinus* possess *in vitro* anti leishmanial activity against *L. major* promastigotes and amastigotes. The crude methanolic flowers extracts of *C. citrinus* induced production of nitric oxide (NO) by infected BALB/c mice peritoneal macrophages *in vitro*, which suggested that the extracts have an immunotherapeutic potential. The *C. citrinus* crude aqueous and methanolic extracts were less toxic to vero cells as compared to the standard *Leishmania* drugs pentostam and liposomal amphotericin B. Further studies are under way to investigate the anti-leishmanial active fractions in crude aqueous and methanolic flower extracts of *C. citrinus*.

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