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Phytochemical Analysis and Antileishmanial Activity of *Clerodendrum myricoides* and *Salvadora persica* Plant Extracts against *Leishmania major*

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Authors' contributions

This work was carried out collaboratively among all the authors. All authors participated in all aspects of the study from the protocol design to the approval of the final manuscript.

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ABSTRACT

Introduction: *Clerodendrum myricoides* and *Salvadora persica* have been reported in management of leishmaniasis which is one of the neglected tropical diseases. The study aimed at undertaking phytochemical analysis as well as investigating the *in vitro* antileishmanial activity of *Clerodendrum myricoides* and *Salvadora persica* extracts on promastigote and amastigote forms of *Leishmania major*.

Materials and Methods: Solvent extraction of the stem parts of the two plants was performed using water, methanol, petroleum ether, dichloromethane and ethyl acetate. Minimum inhibitory concentration (MIC), anti-amastigote and nitric oxide production assays were carried out to demonstrate antileishmanial activity of these plant extracts against the two forms of *Leishmania major* parasite species: promastigote and amastigote. Cytotoxicity assay was then conducted to assess their safe use as herbal medicinal products.

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Results: Both plants showed presence of alkaloids, terpenoids, phenols, anthraquinones and saponins. The *Clerodendrum myricoides* water extract demonstrated the best potential antileishmanial activity against *Leishmania major* promastigotes (MIC=625 µg/ml). The dichloromethane and petroleum ether extract were nontoxic to vero cells but showed moderate to weak activity against *Leishmania major* promastigotes (MIC=1250 µg/ml; 2500 µg/ml) and amastigotes respectively. The *Salvadora persica* ethyl acetate, successive methanol and dichloromethane extracts recorded the most potential activity towards both *Leishmania major* promastigotes and amastigotes.
Conclusions: All *Salvadora persica* and *Clerodendrum myricoides* extracts have potential antileishmanial activity and may offer a cheaper alternative to the more expensive pentavalent antimonials.

Keywords: Leishmaniasis; Clerodendrum myricoides; Salvadora persica; Leishmania major.

1. INTRODUCTION

Leishmaniasis is a vector-borne disease which is as one of the neglected tropical diseases [1,2]. It is endemic in various tropical and subtropical regions, and has also become common in the European Mediterranean area. Globally, 12 million people are reported to be affected with cases from over 98 countries in different continents including South America, Africa, southern Europe and Asia. The statistics are thought to represent about 30% of the cases since many cases go unreported [3,4]. New cases of leishmaniases are estimated to be close to 2 million per year while the population estimated to be at risk worldwide is 350 million [5]. The cause of cutaneous leishmaniasis is *Leishmania major* yet its treatment remains an uphill challenge. Given that there is still no vaccine available for use in humans, chemotherapy has been the main method of treatment. The current recommended first line of drugs require long courses of medication and parenteral administration which have adverse side effects. The cost of treatment is also considered quite expensive in resource-limited areas [6–8]. Recently, the problem of drug resistance has emerged in many endemic areas [9]. The need to develop alternative treatments which are safer, less toxic, less expensive and readily available is required.

It is reported that approximately 50% of the world's population use traditional medicine for their health care [10]. Medicinal plants are virtually used as a primary source of healthcare for a wide variety of illnesses and body conditions [11–16]. However, many medicinal plant have not been scientifically validated for their medicinal properties and thus results in a knowledge and literature gap. In this study, a total of twelve solvent extracts from *Clerodendrum myricoides* (Hoscht) Vatke (Lamiales: Lamiaceae) and *Salvadora persica* L. (Brassicales: Salvadoraceae) were used for preliminary screening of antileishmanial activity against the promastigote and amastigote forms of *Leishmania major*. The selection of these plants was based on their ethnopharmacological use. *Clerodendrum myricoides* roots are used in the management of respiratory infections, arthritis, malaria, tonsillitis, eye infections, and gonorrhea while *Salvadora persica* roots/root-barks are used in the treatment of bacterial and fungal infections such as stomach aches, constipation, back aches, joint aches, body pains and sexually transmitted infections and were shown to accelerate wound healing. Some of the reported infections treated by these plants, such as viral, bacterial, fungal and protozoal infections, have a similar pathogenic mechanism similar to leishmaniasis [17,18].

2. MATERIALS AND METHODS

2.1 Study Site and Design

The study was carried out in the Leishmania laboratory of the Centre for Biotechnology Research and Development (CBRD) situated at the Kenya Medical Research Institute (KEMRI), Nairobi. The *in vitro* studies were carried out using a comparative study design. The efficacy and toxicity of the samples were compared with those of Pentostam® and Amphotericin B®. Schneider's insect media was used as a negative control in experimental chemotherapeutic studies.

2.2 Plant Collection and Extract Preparation

The stems parts of the plants were collected from the leeward side of Ngong, Kenya: *Clerodendrum myricoides* (voucher specimen number VNN2013/01) – 29.4Km SW of Nairobi;

Salvadora persica (voucher specimen number VNN2013/02) – 32.9Km SW of Nairobi. These were identified for authentication by taxonomists of the Botany Department Herbarium of the University of Nairobi. They were then kept in the KEMRI laboratory where the study was done. The plant extracts were processed according to a previously published method [19,20]. Briefly, the stems and barks were cut into small pieces and air-dried for 14 days under a shed. The dried specimens were ground using an electrical mill in readiness for extraction. Total extracts of methanol and water were the first to be prepared.

100 g of each powder was soaked in absolute methanol for 24 hours. The extract was filtered, dried with Na_2SO_4 and the solvent removed under vacuum in a rotary evaporator at 30-35ºC. For aqueous extraction, 100 g of ground material in 600ml of water was placed in a water bath and maintained at 60ºC for 2 hours. The filtrate was freeze-dried, weighed and stored at -20ºC until required for use. The extracts were tested for cytotoxicity and *in vitro* anti-parasitic activity against *Leishmania major* parasites. Cold sequential extraction was carried out on plant material with distilled organic solvents of increasing polarity (petroleum ether > dichloromethane > ethyl acetate > methanol). A volume of 600ml of petroleum ether was added and flasks placed on a shaker and soaked for 48 hours. The residue was filtered using a Buchner funnel under vacuum until the sample dried. The sample was soaked further with 600ml of petroleum ether for 24 hours until the filtrate remained clear. The filtrate was then concentrated under vacuum by rotary evaporation at 30-35ºC as previously described [21]. The concentrate was transferred to a sample bottle and dried under vacuum; the weight of the dry extract was recorded and stored in universal bottles at a temperature of -20ºC until required for bioassay. The process was repeated sequentially for dichloromethane, ethyl acetate and methanol. The yield percentage of each extract was calculated as follows:

$$\text{Percentage yield of extract} = \frac{\text{Final weight of dried extract}}{\text{Initial weight of powder}} \times 100$$

The extracts were screened for cytotoxicity and *in vitro* anti-leishmanial activity.

2.3 Phytochemical Studies

The plant extracts were screened for the presence of different phytochemicals as previously described [22]. Briefly, each sample extract was diluted with its solvent. A volume of 3 µl to 6 µl of diluted sample of the test extracts was spotted one inch from the bottom and an inch from the left hand margin of a pre-coated thin layer chromatography plate. Correct labeling was ensured to enable observation of the results. When the spots were dry, the plates were developed in selected mobile solvent phases. Sufficient solvent was poured into a chromatography jar to a level of half-inch from the bottom. The spotted plates were mounted vertically in the jars which were paper lined to saturate the atmosphere inside with the solvent phase. The mounting was such that the extract spots were just above the solvent level. The jars were covered with greased lids and allowed to develop. As the solvent rose by capillarity, ascending chromatographic separation was obtained, resulting in discrete spots. When the solvent front reached a point one inch below the top of each plate, the plate was removed from the jar and allowed to dry. The separated spots were located by fluorescence under ultraviolet light.

2.3.1 Terpenoids

Terpenoids were located by spraying plates with vanillin sulphuric acid and heating in a pre-heated oven at 110ºC. Presence of terpenoids was determined by different spots observed with different colours.

2.3.2 Alkaloids

Plates were sprayed with Dragendoff reagent. Presence of alkaloids was determined by orange, brown or yellow background spots, or intense yellow colour in white background.

2.3.3 Phenols

Plates were sprayed with 1% ferric chloride and 1% potassium ferricyanide. Blue spots were observed to determine presence of phenols.

2.3.4 Anthraquinones

Plates were sprayed with methanolic potassium hydroxide. Orange or yellow colours were observed, indicating presence of anthraquinones.

2.3.5 Saponins

To test presence of saponins, a solution of the extract in a little water was shaken vigorously in

a test tube. Any foaming indicated presence of saponins.

2.4 Experimental Animals

Eight weeks old BALB/c mice for macrophage assays were obtained from KEMRI's animal house. All animals were of the same sex and uniform size. The experiments were done in compliance with Animal Care and Use Committee (ACUC) guidelines of KEMRI.

2.5 Leishmania Parasites

Leishmania major strain (Strain IDU/KE/83=NLB-144), originally isolated from a female *P. duboscqi* [23], maintained by cryopreservation, in vitro culture and periodic passage in BALB/c mice. Parasites were cultured in Schneider's Insect Medium (SIM) supplemented with 20% heat-inactivated foetal bovine serum, 100 µg/ml penicillin G and 100µg/ml streptomycin [24,20] and 500 µg/ml 5-fluorocytosine arabinioside [25]. The cultures were made in T25 sterile disposable culture flasks (25ml) and incubated at 25ºC and grown to stationary phase to generate infective metacyclic promastigotes [26]. Pentostam® was used as a positive control. The metacyclic promastigotes were isolated by negative selection using peanut agglutinin [27].

2.6 Preparation of the Test Drugs

Stock solutions of the fractions were made in culture media for antileishmanial assays and re-sterilized by filtering through 0.22 µm filter flasks in a laminar flow hood. If some of the extract was found not to dissolve easily in water or media, they were first dissolved in 1% dimethyl sulfoxide (DMSO) to avoid solvent carry-over [28]. All prepared drugs were stored at 4ºC and retrieved only during use.

2.7 Bioassays

2.7.1 MIC (Minimum Inhibitory Concentration) anti-promastigote assay

Leishmania major promastigotes (1 x 10^6 parasites/ml) were grown and incubated in Schneider's Insect Medium (SIM) culture media containing different concentrations of the test extracts ranging from 1 mg/ml to 1 µg/ml. Cell growth was evaluated by assessment of visibility turbidity in order to evaluate MIC. The lowest concentration of the samples that prevented growth was considered as the MIC.

2.7.2 Anti-amastigote assay

This was carried out as previously described [29]. Briefly, peritoneal macrophages were obtained from BALB/c mice. The mice were injected with 2% starch solution to stimulate macrophage proliferation. After 24 hours, they were anaesthetized using 100 µl pentobarbital sodium (Sagatal®). The body surface was disinfected with 70% ethanol. The torso skin was torn dorsoventrally to expose the peritoneum. Using a sterile syringe and needle, 10 ml of sterile cold phosphate-buffered saline (PBS) was injected into the peritoneum. After shaking the mouse, peritoneal macrophages were harvested by withdrawing the PBS. The contents were transferred into a sterile 50ml centrifuge tube. The suspension was centrifugally washed at 2000 rpm for 10 minutes and the pellet resuspended in complete RPMI 1640 medium.

Macrophages were adsorbed in 24-well plates and allowed to adhere for 4 hours at 37ºC in 5% CO_2. Non-adherent cells were washed with cold PBS and the cultures incubated overnight in RPMI. Adherent macrophages were infected with a parasite/macrophage ratio of 6:1 and further incubated at 37ºC in 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 a positive control drug for comparison of parasite inhibition. The medium and drug were replenished daily for 3 days. After 5 days, the monolayers were washed with PBS at 37ºC, fixed in methanol and stained with Geimsa. The number of amastigotes was determined by counting at least 100 macrophages in duplicate cultures, and the results expressed as infection rate (IR) and multiplication index (MI) [30] as follows:

$$\text{IR} = \text{No. of infected macrophages in 100 macrophages}$$

$$\text{MI} = \frac{\text{No. of amastigotes in experimental culture /100 macrophages}}{\text{No. of amastigotes in control culture /100 macrophages}} \text{ x } 100$$

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

2.7.3 Nitric oxide production assay

Nitric oxide release in macrophage cultures was measured using the Greiss reaction for nitrites

[31]. Briefly, 100 µl of the supernatants was collected 48 hours after introducing the test drug into the culture medium. The assay was done in triplicate wells in 96-well microtiter plates. To this, 60 µl of Greiss Reagent A (1% sulphanilmide in 1.2M HCl) was added followed by 60 µl of Greiss Reagent B (0.3% N-(1-naphthyl) ethylenediamine). The absorbance was measured at 540nm in an enzyme-linked immunosorbent assay (ELISA) reader. Nitrite (NO_2^-) was calculated from a standard curve constructed using sodium nitrite in RPMI.

2.7.4 Cytotoxicity studies

VERO cells were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10% FBS. The cells were cultured at 37ºC in 5% CO2 for 24 hours by trypsinization, pooled in 50ml vial and in 100 µl cells suspension (1 x 10^6 cells/ml) put into 2 wells of rows A-H in a 96-well microtiter plate for one sample to attach. The medium was aspirated off and 150 µl of the highest concentration (1000 µg/ml) of each of the test samples at 562 nm was added into the same row and serially diluted. The experimental plates with the cells were incubated further at 37ºC for 48 hours. The controls used were cells with no extract, medium alone. 10 µl MTT reagent was added into each well and the cells incubated for 2-4 hours until a purple precipitate was visible under a microscope. The medium together with MTT were aspirated after which 100µl of DMSO was added and plates shaken for 5 minutes. The absorbance was measured for each well at 562nm using a micro-titer plate reader [32]. The results were expressed as the concentration at which the extract inhibited 50% growth of the cells (IC_{50}).

2.8 Statistical Analysis

All experiments were done in triplicate. Statistical analysis of the differences between mean values obtained from the test extracts compared to the controls was done by student's t-test. ANOVA was used to determine the differences between the various test extracts. A probability value of $p \leq 0.05$ was considered to be statistically significant. Analysis software program, Chemosen, available at KEMRI was used to determine IC_{50} concentrations.

3. RESULTS AND DISCUSSION

Detailed Solvent extraction was used to prepare plant extracts due to its ease of use, efficiency and wide applicability. The stems parts of *Clerodendrum myricoides* and *Salvadora persica* were successfully extracted with water, methanol, petroleum ether, dichloromethane and ethyl acetate. The water extract was brownish in colour and powdery in consistency. The methanol extract was greenish in colour and oily in consistency. The petroleum ether extract was yellowish in colour and oily in consistency. The dichloromethane and ethyl acetate extracts were greenish and brownish in colour and resinous in consistency.

Higher yield of extracts was obtained from solvents with high polarity than those with low polarity as shown in Table 1. The crude extracts (water and methanol) had higher yields followed by successive extracts. This could be influenced by the type of solvent used for extraction, which also plays a significant role in the solubility of active compounds contained in the plant extracts. This in turn may influence the antileishmanial activities of these extracts [33].

Thin-layer chromatography (TLC) visualization reagents aided in screening for the presence of different classes of secondary metabolites in *Clerodendrum myricoides* and *Salvadora persica* extracts. From the results tabulated in Table 2, all *Clerodendrum myricoides* plant extracts tested positive for more than one phytochemical component. The water extract showed the strongest presence of all phytochemical components tested: alkaloids; terpenoids; phenols; anthraquinones and saponins. The dichloromethane and petroleum ether extracts showed strong presence of most phytochemicals, except saponins. The ethyl acetate extract showed good presence of all phytochemicals, except saponins. The total methanol extract showed weak presence of all phytochemicals, with absence of alkaloids and saponins. The successive methanol extract showed the weakest presence of phytochemicals with absence of all except anthraquinones.

From Table 3, all *Salvadora persica* plant extracts tested positive for more than one phytochemical component. The water extract showed good presence of all phytochemical components tested: alkaloids; terpenoids; phenols; anthraquinones and saponins. The dichloromethane, ethyl acetate and petroleum ether extracts showed the strongest presence of most phytochemicals respectively, except saponins. The total methanol extract showed good presence of most phytochemicals, except saponins. The successive methanol extract showed the least presence of phytochemicals, with absence of anthraquinones and saponins.

Table 1. Percentage yield of solvent extracts of the ground plant materials

Test plants	% Yield of extracts (g)					
	Water	TM	PE	DCM	EA	SM
Clerodendrum myricoides	10.85	5.21	0.30	0.19	0.09	4.72
Salvadora persica	7.98	2.75	0.19	0.44	0.13	2.48

TM-Total Methanol, PE-Petroleum ether, DCM-Dichloromethane, EA-Ethyl acetate, SM-Successive Methanol

Table 2. Phytochemical screening of six solvent extracts of *Clerodendrum myricoides*

Phytochemicals	Test extracts					
	Water	TM	PE	DCM	EA	SM
Alkaloids	+++	-	++	+++	++	-
Terpenoids	+++	+	+++	+++	++	-
Phenols	+++	+	+++	+++	+++	-
Anthraquinones	+++	+	++	+++	++	+
Saponins	++	-	-	-	-	-

TM-Total Methanol, PE-Petroleum ether, DCM-Dichloromethane, EA-Ethyl acetate, SM-Successive Methanol
Key: +++ very deep colours with different spots observed; ++ medium-coloured spots observed
+ faint-coloured spots observed; - absent

Table 3. Phytochemical screening of six solvent extracts of *Salvadora persica*

Phytochemicals	Test extracts					
	Water	TM	PE	DCM	EA	SM
Alkaloids	++	++	+++	+++	+++	++
Terpenoids	++	++	+++	+++	+++	+
Phenols	++	+++	+++	+++	+++	++
Anthraquinones	+++	+++	++	+++	+++	-
Saponins	+++	-	-	-	-	-

TM-Total Methanol, PE-Petroleum ether, DCM-Dichloromethane, EA-Ethyl acetate, SM-Successive Methanol
Key: +++ very deep colours with different spots observed; ++ medium-coloured spots observed;
+ faint-coloured spots observed; - absent

Several studies have reported on the activity of alkaloids on *Leishmania major* [34-37]. Antileishmanial activity of terpenoids on *Leishmania major* has also been reported [38]. Antileishmanial activity of phenols on *Leishmania major* have also been reported [39]. Anthraquinones have reported antileishmanial activity [40]. Studies on saponins suggesting antileishmanial activity are also reported [41–43]. The presence of these compounds may have attributed to the antileishmanial activity of the extracts, either synergistically or individually.

Preliminary antileishmanial activity screening of plant extracts is usually done using the two forms of *Leishmania* parasite: promastigote and amastigote forms. Assays using the promastigote form are easy to perform due to their easy cultivation and maintenance. The above notwithstanding, they usually indicate possible antileishmanial activity of the test drug and therefore preliminary screening of drugs using promastigotes needs to be complemented with assays performed using amastigotes [40,20]. Preliminary antileishmanial screening of *Clerodendrum myricoides* and S*alvadora persica* extracts performed on the promastigote form of *Leishmania major* showed that their activity against promastigotes is not in relation to their polarity. Polar water and total methanol extracts of *Clerodendrum myricoides* were the most active with MIC values of 625 µg/ml. The *Salvadora persica* ethyl acetate extract showed the most activity with an MIC value of 625 µg/ml.

The *Clerodendrum myricoides* water and total methanol extracts were the most active with MIC values of 625 µg/ml (Fig. 1). This was comparable to the standard Pentostam® and Amphotericin B® anti-leishmanial drugs ($p<0.05$). Amphotericin B showed MIC value of 12.5 µg/ml and Pentostam® showed MIC value of 25 µg/ml. The dichloromethane extract showed MIC value of 1250 µg/ml. The petroleum ether, ethyl acetate and successive methanol showed the least activity with MIC values of 2500 µg/ml.

Schneider's Insect Medium (SIM) was also considered the negative control. The *Leishmania major* parasites continued dividing and proliferating in the media. Thus no anti-leishmanial activity against the growth of parasites was recorded.

The *Salvadora persica* ethyl acetate extract showed the most activity with MIC value of 625 µg/ml (Fig. 2). This was also comparable to the standard Pentostam® and Amphotericin B® anti-leishmanial drugs ($p<0.05$). Amphotericin B showed MIC value of 12.5 µg/ml and Pentostam® showed MIC value of 25 µg/ml. The dichloromethane and successive methanol extracts showed MIC values of 1250 µg/ml. The water, total methanol and petroleum ether extracts showed the least activity with MIC

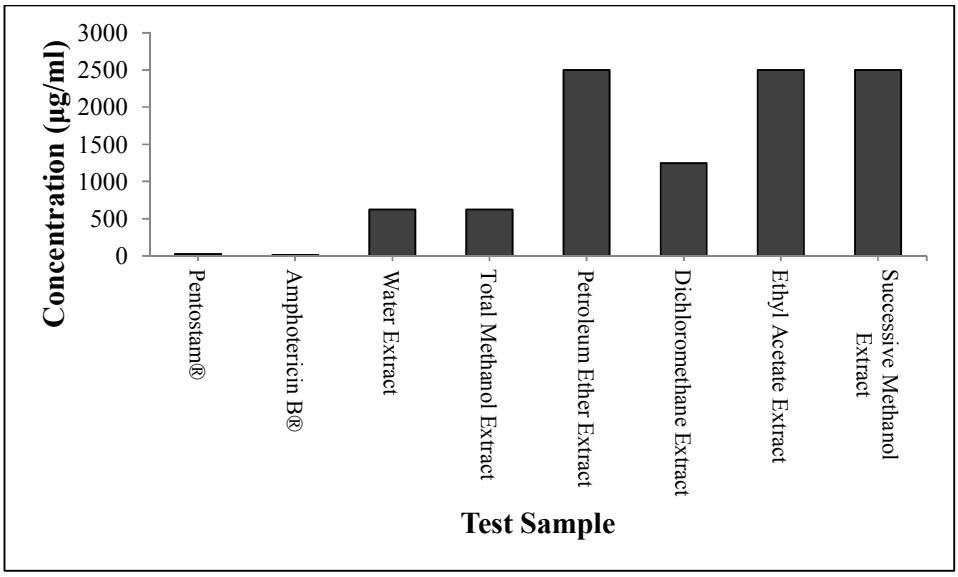

Fig. 1. Minimum inhibitory concentration (MIC) values of six solvent extracts of *Clerodendrum myricoides* compared to commercial standard drugs

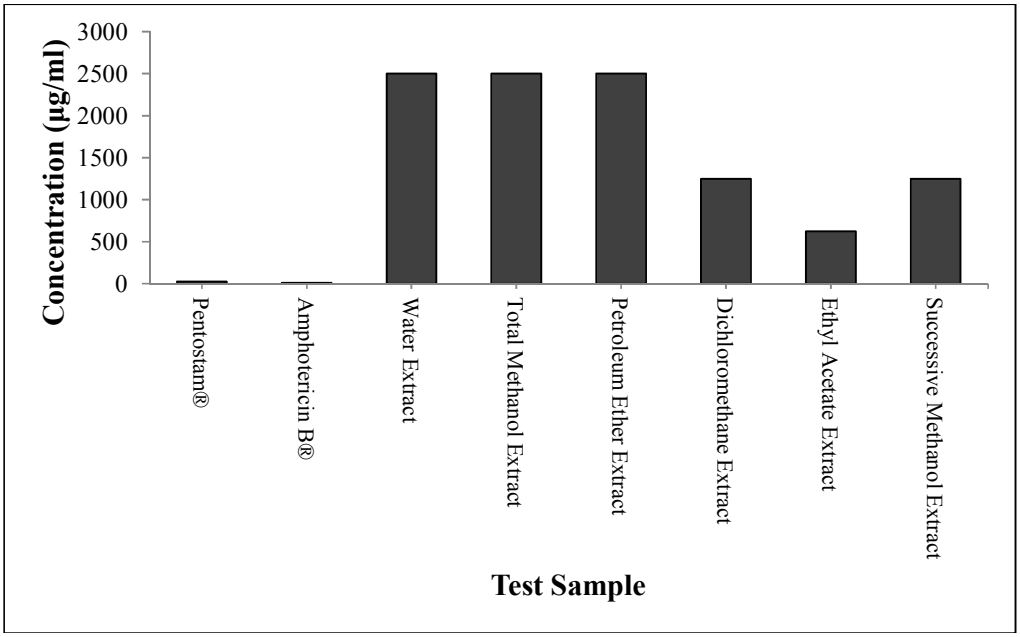

Fig. 2. Minimum inhibitory concentration (MIC) values of six solvent extracts of *Salvadora persica* compared to commercial standard drugs

values of 2500 µg/ml. Schneider's Insect Medium (SIM) was considered the negative control. The *Leishmania major* parasites continued dividing and proliferating in the media. Thus no anti-leishmanial activity against the growth of parasites was recorded.

To perform anti-amastigote assays, the promastigote form of the parasite must transform into the amastigote form. To achieve this, promastigotes are transformed into amastigotes intracellularly by infecting macrophages of monocytic cell lines derived from mice [44]. The activity of these extracts on macrophages infected with the amastigote form of *Leishmania major in vitro* was seen to be dose-dependent as shown in Fig. 3. Higher concentrations of the extracts seemed to reduce the infection rate of macrophages.

At their highest concentrations (200 µg/ml), the dichloromethane, water and successive methanol extracts of *Clerodendrum myricoides* were found to be more active than petroleum ether, total methanol and ethyl acetate extracts. This trend though did not translate to their multiplication indices. The successive methanol extract, at a concentration of 200 µg/ml, reduced the number of parasites by 91.32% effectively compared to the standard reference drug Pentostam ($p<0.05$). This low MI value could be as a result of the successive methanol extract killing the parasites and inhibiting the parasite growth [45].

The petroleum ether, total methanol, ethyl acetate, dichloromethane and water extracts also reduced the number of parasites though they were not significantly different to the standard reference drugs. The dichloromethane extract recorded the least parasite reduction activity at all concentrations. Its MI value was 88.95%, 89.47% and 88.95% at 200 µg/ml, 100 µg/ml and 50 µg/ml concentrations respectively. This reduced the number of parasites by 11.05%, 10.53% and 11.05% respectively when compared to the negative control, RPMI ($p<0.05$). There was no significant difference in the activity of the water and successive methanol extracts at concentration of 200 µg/ml with Pentostam® at a concentration of 50 µg/ml ($p>0.05$).

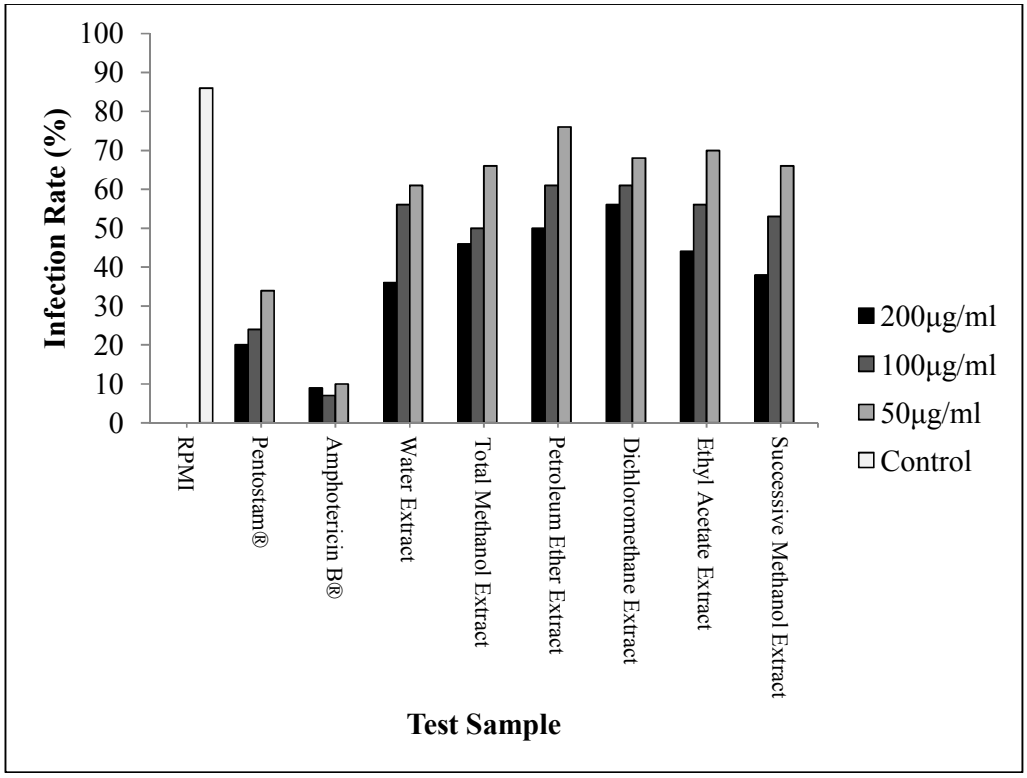

Fig. 3. Infection rates of infected BALB/c macrophages treated with six solvent extracts of *Clerodendrum myricoides* compared to commercial standard drugs

Interestingly, all *Salvadora persica* extracts at their highest concentrations (200 µg/ml) had IR values not significantly different to the standard drug Pentostam at concentration of 50 µg/ml ($p>0.05$) as shown in Fig. 4.

In relation to their parasite growth reduction, the ethyl acetate and successive methanol extracts recorded MI values of 9.34% and 10% respectively at a concentration of 200 µg/ml. These reduced the parasites by 90.66% and 90% respectively when compared to the negative control, RPMI ($p<0.05$). The extracts were significantly better than Pentostam®, which recorded a value of 13.16% at a concentration of 200 µg/ml ($p<0.05$). This could also be as a result of the extracts killing the parasites and inhibiting the parasite growth [45]. The water extract recorded the least parasite reduction activity at concentrations of 100 µg/ml and 50 µg/ml. Its MI value was 78.95% and 78.03% at 100 µg/ml and 50 µg/ml concentrations respectively. This reduced the number of parasites by 21.05% and 21.97% respectively when compared to the negative control, RPMI ($p<0.05$).

The macrophage-based amastigote assay is also a good indicator for drugs that may have immunostimulatory properties. Macrophages play an important role in the killing of *Leishmania* parasites and control of leishmaniasis. Infected macrophages elicit a killing mechanism that involves the production of nitric oxide. This is measured using the Greiss assay. Nitric oxide is therefore a key signaling molecule in host defense mechanism against intracellular pathogens [46–49]. Its production as an important step in antileishmanial activity in murine macrophages has been documented [50–54]. Infected macrophages treated with *Clerodendrum myricoides* successive methanol extract (Fig. 5) produced 9.32% (14.19 µM) more nitric oxide than non-treated macrophages (12.98 µM) at concentration of 50 µg/ml. This was not significantly different to those treated with Pentostam® and Amphotericin B® which recorded nitrite values of 14.35 µM and 14.13 µM respectively at concentration of 200 µg/ml ($p>0.05$). This extract showed good antileishmanial activity against the amastigotes reducing the number of parasites by 90%.

This data suggests that the *Clerodendrum myricoides* successive methanol extract contains compounds that stimulate the production of nitric oxide by infected macrophages. This in turn activates the killing action of NO against

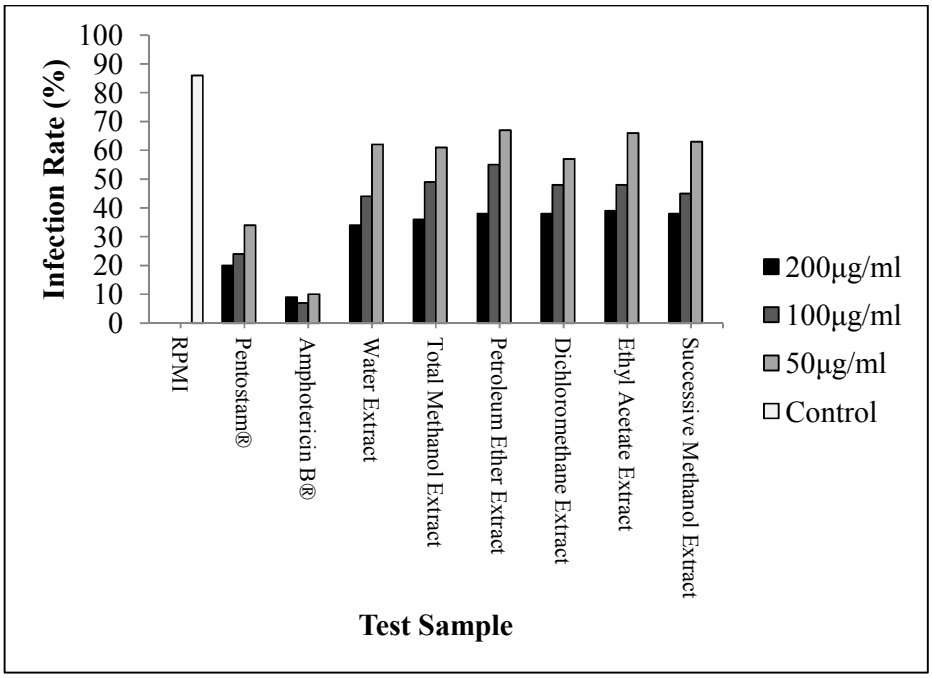

Fig. 4. Infection rates of infected BALB/c macrophages treated with six solvent extracts of *Salvadora persica* compared to commercial standard drugs

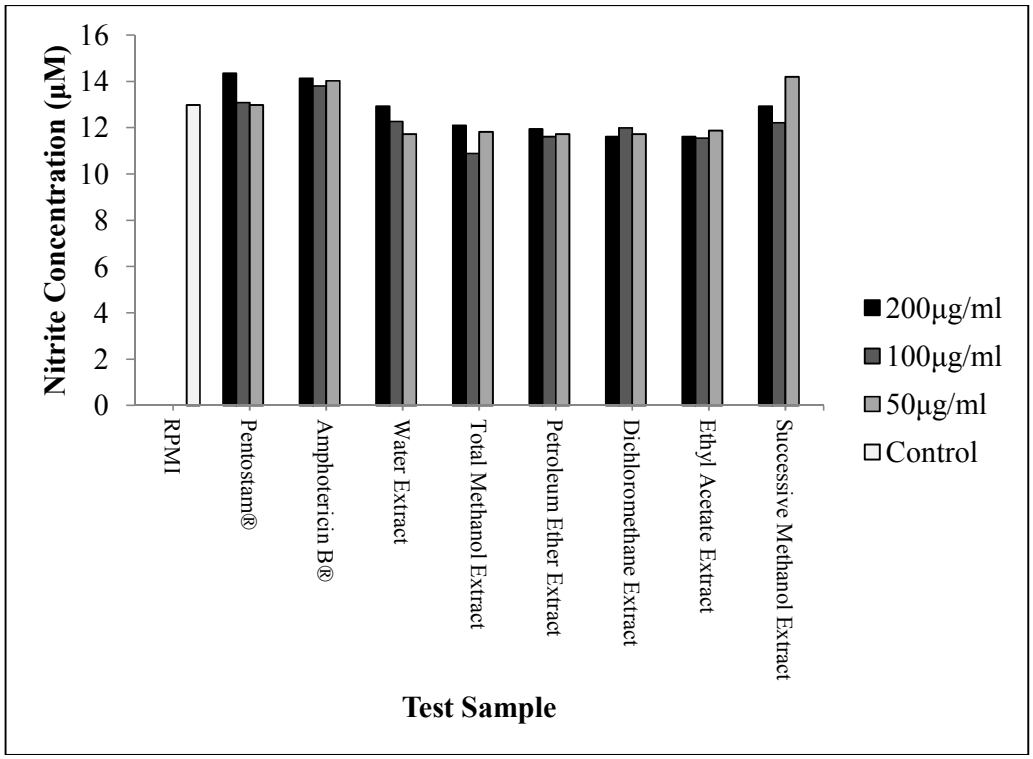

Fig. 5. Nitric oxide produced by infected BALB/c macrophages treated with six solvent extracts of *Clerodendrum myricoides* compared to commercial standard drugs

Leishmania major parasites [55]. The total methanol extract produced the least amount of nitrite at concentration of 100 µg/ml when compared to Pentostam®, Amphotericin B® and RPMI ($p<0.05$). Infected macrophages treated with *Clerodendrum myricoides* water, petroleum ether, dichloromethane and ethyl acetate extracts produced nitrite values significantly lower than Pentostam®, Amphotericin B® and RPMI ($p<0.05$). This suggests that their mechanism of action against *Leishmania major* parasites does not involve nitric oxide. Its action could involve either killing the parasites directly or inhibiting the parasite growth.

Infected macrophages treated with the *Salvadora persica* total methanol extract (Fig. 6) at a concentration of 200 µg/ml produced 5.55% (13.70 µM) more nitric oxide than non-treated macrophages (12.98 µM). This was not significantly different to those treated with Pentostam® and Amphotericin B® at the same concentration ($p>0.05$). The extract also showed good antileishmanial activity against amastigotes with an infection rate of 36%.

The association between the amastigotes and infected macrophages was reduced by 61.58%. This suggests that the *Salvadora persica* total methanol extract also contains compounds that stimulate the production of nitric oxide by infected macrophages. Therefore, this activates the killing mechanism of action of NO against *Leishmania major* parasites. Despite the good antileishmanial activity of ethyl acetate and successive methanol extracts of *Salvadora persica* against amastigotes, they did not show increased levels of NO (11.44 µM and 11.33 µM respectively) compared to the control (12.98 µM). The water and petroleum ether extracts produced nitrite values significantly lower than Pentostam®, Amphotericin B® and RPMI ($p<0.05$) with the dichloromethane extract recording the least amount of nitric oxide at concentration of 100µg/ml when compared to Pentostam®, Amphotericin B® and RPMI ($p<0.05$). Therefore, this suggests that their mechanism of action against *Leishmania major* parasites does not involve nitric oxide. Its action could involve either killing the parasites directly or inhibiting the parasite growth.

To assess the potential toxicity of test plants or drugs, cell based cytotoxicity tests are performed using cultured mammalian cells. The tests therefore indicate the degree of safe therapeutic use of these plants. In this study, the cytotoxic effect of *Clerodendrum myricoides* and *Salvadora persica* extracts was tested on mammalian kidney fibroblast cells (vero cell line) by using MTT assay. This colorimetric assay has been extensively used in cell proliferation and cytotoxicity assays [56]. A progressive decrease in cytotoxicity of *Clerodendrum myricoides* extracts was observed with decreasing polarity as shown in Fig. 7.

Most of its extracts were toxic: total methanol (IC_{50}=0.43 µg/ml); water (IC_{50}=0.58 µg/ml); successive methanol (IC_{50}=0.64 µg/ml); ethyl acetate (IC_{50}=1.81 µg/ml). These observations are in agreement with studies carried out on *Clerodendrum myricoides* that report of its toxicity [57–59]. This suggests that the extracts exude some toxic effects in mammalian cells, thus discouraging its therapeutic use in humans. This could be due to the presence of phenols. Reports on cytotoxicity of phenol compounds have been reported [60–62]. The petroleum ether and dichloromethane extract were the least toxic with IC_{50} concentration values at 2.44 µg/ml and 2.36 µg/ml respectively ($p<0.05$).

Interestingly, extracts of *Salvadora persica* in Fig. 8 showed a progressive decrease in cytotoxicity with increasing polarity. The ethyl acetate (IC_{50}=3.84 µg/ml); total methanol (IC_{50}=3.65 µg/ml); water (IC_{50}=3.54 µg/ml) and successive methanol (IC_{50}=3.25 µg/ml) extracts were not toxic to the growth of vero cells.

These extracts also demonstrated potential antileishmanial activity against *Leishmania major* parasites. This suggests that the extracts are safe for therapeutic use. However, the Petroleum ether and dichloromethane extracts were toxic in mammalian cells. This cytotoxic effect could be due to the strong presence of terpenoids. Terpenoids are reported to exert their cytostatic properties leading to the inhibition of cell growth [63].

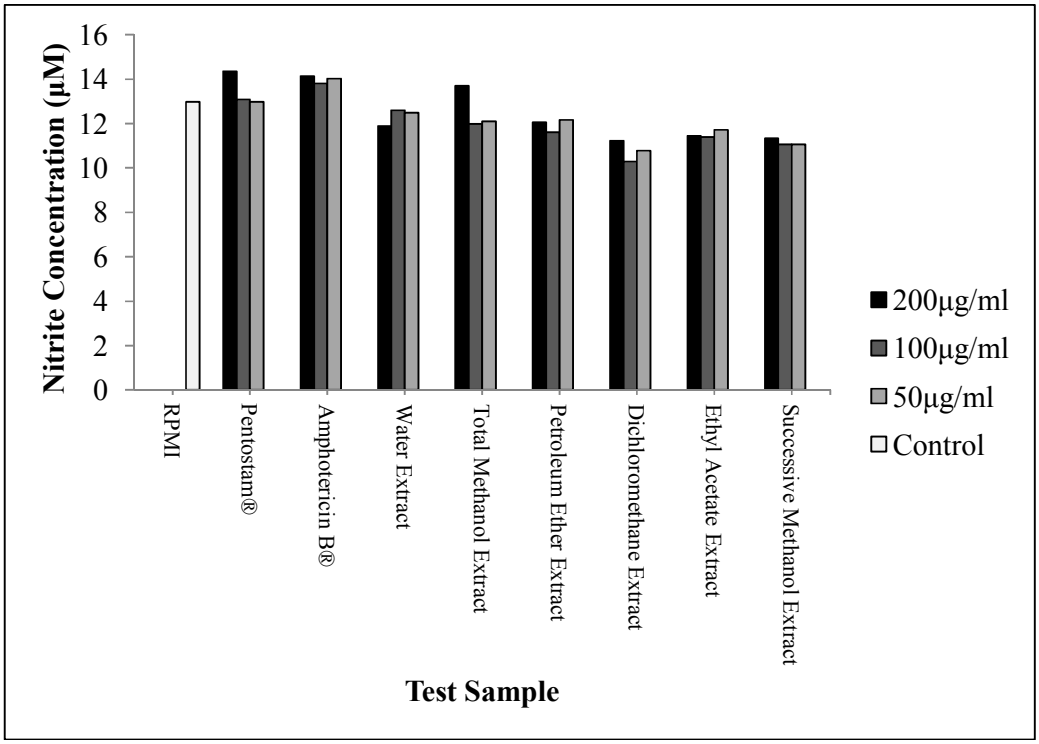

Fig. 6. Nitric oxide produced by infected BALB/c macrophages treated with six solvent extracts of *Salvadora persica* compared to commercial standard drugs

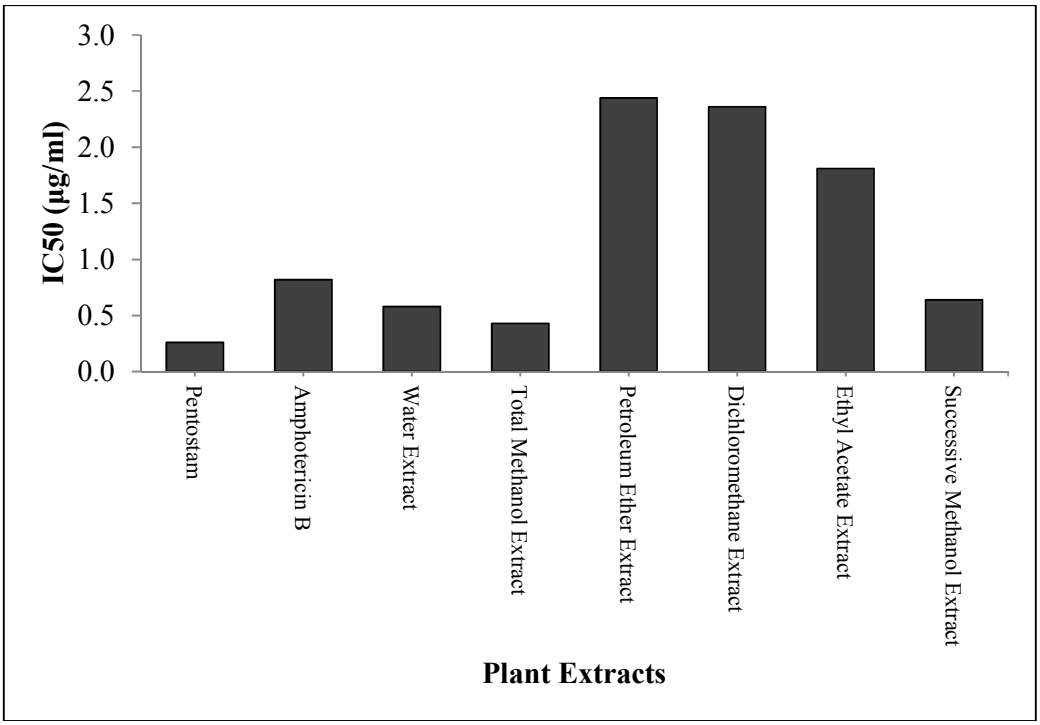

Fig. 7. Inhibitory concentration of six solvent extracts of *Clerodendrum myricoides* against vero cells compared to commercial standard drugs

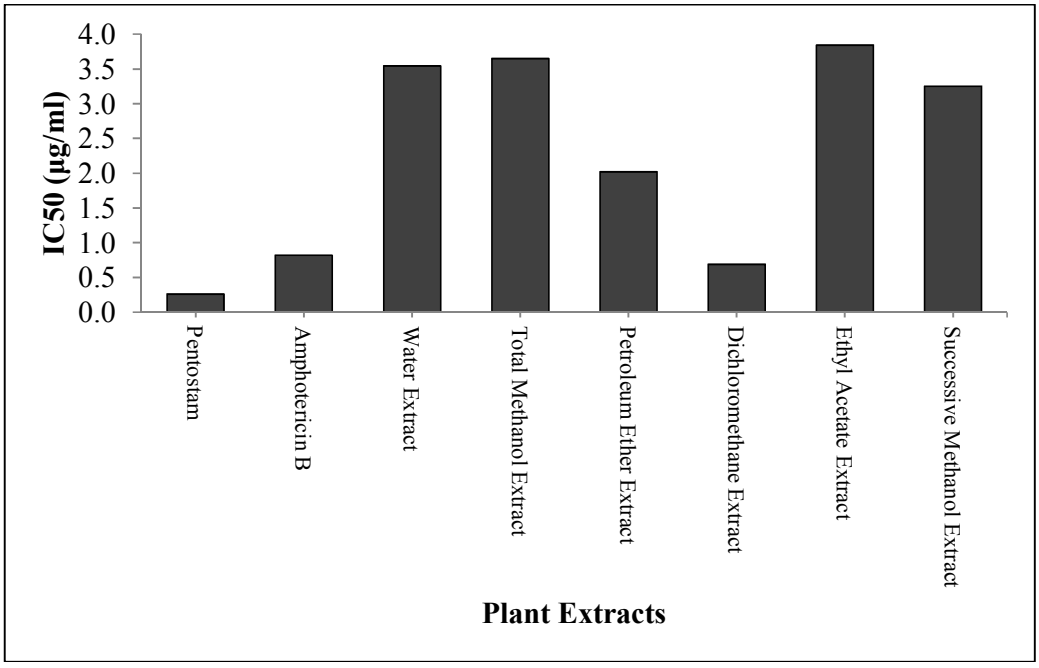

Fig. 8. Inhibitory concentration of six solvent extracts of *Salvadora persica* against vero cells compared to commercial standard drugs

4. CONCLUSION

The results of this study revealed the antileishmanial activity of *Clerodendrum myricoides* and *Salvadora persica*. The phytochemical screening results also shed light on the activity portrayed by these extracts. Solvent extracts of *Clerodendrum myricoides* recorded the most toxicity reports compared to *Salvadora persica*. The *Clerodendrum myricoides* water extract demonstrated the best potential antileishmanial activity against *Leishmania major* promastigotes and amastigotes. The dichloromethane extract showed no toxicity in vero cells, and only showed potential activity against *Leishmania major* promastigotes. All *Salvadora persica* extracts; and all *Clerodendrum myricoides* extracts apart from ethyl acetate and petroleum ether extracts showed potential antileishmanial activity against *Leishmania major* parasites. This could be attributed by the phytochemical constituents present in these extracts. It is evident that the medicinal use of the reported plants for *Leishmaniasis* merit documentation of the traditional knowledge of the Maa community. It is important to document and use this knowledge in producing novel products that could improve healthcare in rural communities. There is also need of greater awareness from scientists and local government for improved preservation of these medicinal plants.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All procedures performed in studies involving animals were in compliance with Animal Care and Use Committee (ACUC) guidelines of the Kenya Medical Research Institute (KEMRI).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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