<u>Research Article</u>

In vitro antileishmanial activity and phytochemical analysis of *Carissa edulis* against *Leishmania major*

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Background: Medicinal plants have been of great importance to many traditional communities for many generations*.* However, there is need to carry out scientific studies in order to confirm the medicinal properties of many plants used traditionally. *Carissa edulis* Forskk. (Gentiales: Apocynaceae) used by local communities for the treatment of various diseases has showed antiviral, antibacterial and antiprotozoal properties although there are no studies demonstrating its antileishmanial activity.

Objective: To investigate *in vitro* antileishmanial activity of extracts of *Carissa edulis* on promastigote and amastigote forms of *Leishmania major*.

Methodology: Solvent extraction of the stem parts of *C. edulis* 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 *C. edulis* extracts against the two forms of *L. major* parasite species: promastigote and amastigote. The extracts were also screened for phytochemical constituents present. Cytotoxicity assay was then done to assess their safe use as herbal medicinal products.

Results: The *C. edulis* petroleum ether extract showed the strongest antileishmanial activity against *L. major* promastigotes (MIC=625μg/ml) with the water, dichloromethane and ethyl acetate extracts recording the weakest activity (MIC=2500μg/ml). The successive methanol extract reduced the number *L. major* amastigotes by 88.29% compared to the negative control (RPMI). The water (13.37μM), petroleum ether (12.93μM) and successive methanol extracts (12.82μM) produced nitrite values lower than the standard drugs Pentostam® (14.35μM) and Amphotericin B (14.13μM).

Discussion: All *C. edulis* extracts have potential antileishmanial activity against *L. major*. Preliminary phytochemical screening of these extracts showed presence of alkaloids, terpenoids, phenols, anthraquinones and saponins. These phytochemicals were previously reported to have antileishmanial activity. Therefore, the plant extracts could offer an opportunity to develop cheaper antileishmanial alternatives to the more expensive pentavalent antimonials.

Key words: *C. edulis*, *L. major*, promastigote, amastigote

1. Introduction

Leishmaniasis is a vector-borne disease classified as one of the neglected tropical diseases amongst schistosomiasis, soil-transmitted helminth (STH) infections, lymphatic filariasis (LF), human African trypanosomiasis (HAT), leprosy, trachoma and onchocerciasis (Hotez et al*,* 2007; Boelaert and Sundar,

2013). The disease is endemic in numerous tropical and subtropical areas, and is also gaining ground in the European Mediterranean area. An estimated 12 million people are currently affected worldwide with cases of the disease reported from 98 countries in South America, Africa, southern Europe and Asia. This figure is thought to represent only 30% of the cases since the remaining cases go unreported (Desjeux, 2004; Alvar et al, 2012). 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 (World Health Organization, 2010). *Leishmania major* is the causative agent of cutaneous leishmaniasis and its treatment remains a 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 (Guerin et al, 2002; Dube et al, 2005; Singh, 2006). Recently, the problem of drug resistance has emerged in many endemic areas (Croft, Sundar, and Fairlamb, 2006). There is need to develop alternative treatments which are safer, less toxic, less expensive and readily available.

According to the World Health Organization (WHO), around 40% to 60% of the world's people turn to traditional medicine for their health care (Dutta et al, 2007). Medicinal plants have been of great importance to many traditional communities for generations. They are virtually used as a primary source of healthcare for a wide variety of illnesses and body conditions (Ateş and Erdoğrul, 2003; Dulger et al, 2005; Nair, 2005; Şengül et al, 2005; Kumar et al, 2006; Mathabe et al, 2006). However, there is still a lack of experimental scientific studies confirming the medicinal properties of many of these remedies. *In vitro* antileishmanial screening methods furnish the preliminary observations that are necessary to select, among the plant products, those with potentially useful activities for further chemical and pharmacological studies. In this study, six solvent extracts of *Carissa edulis* were used for preliminary screening of antileishmanial activity against the promastigote and amastigote forms of *Leishmania major*. The selection of *Carissa edulis* was based on data obtained from literature, traditional uses of the plant and chemical composition of the plant or other plants belonging to the same genus. Some of the reported infections treated by this plant, such as viral, bacterial, fungal and protozoal infections, have a similar pathogenic mechanism similar to leishmaniasis (Croft and Coombs, 2003; Donald, 2003).

2. Methodology

2.1 Study Site

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.

2.2 Study Design

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.

2.3 Plant Collection and Extract Preparation

The stems parts of *Carissa edulis* (voucher specimen number VNN2013/03) were collected from the leeward side of Ngong, Kenya - 25.4Km 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 the method of Kingondu et al, (2009). 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.

100g 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, 100g 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 *L. 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 described by Harborne (1994). 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 then calculated. The extracts were screened for cytotoxicity and *in vitro* anti-leishmanial activity.

2.4 Phytochemical Studies

The plant extracts were screened for the presence of different phytochemicals as described by Harborne (1998). Briefly, each sample extract was diluted with its solvent. A volume of 3μl to 6μl 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.

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.

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.

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

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

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.

2.5 Experimental Animals

Eight week 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.

2.6 Leishmania Parasites

Leishmania major strain (Strain IDU/KE/83=NLB-144), originally isolated from a female *P. duboscqi* (Beach et al, 1984), 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, 100µg/ml penicillin G and 100µg/ml streptomycin (Hendricks and Wright, 1979) and 500µg/ml 5-fluorocytosine arabinioside (Kimber et al, 1981). 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 (Evans et al, 1989). Pentostam® was used as a positive control. The metacyclic promastigotes were isolated by negative selection using peanut agglutinin (Tonui et al, 2004).

2.7 Preparation of the Test Drugs

Stock solutions of the fractions were made in culture media for antileishmanial assays and re-sterilized by filtering through 0.22µm 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 (Dorin et al, 2001).

All prepared drugs were stored at 4ºC and retrieved only during use.

2.8 Bioassays

2.8.1 MIC (Minimum Inhibitory Concentration) Anti-Promastigote Assay

Leishmania major promastigotes (1×10^6 parasites/ml) were grown and incubated in Schneider's Insect Medium (SIM) culture media containing different concentrations of the test extracts ranging from 1mg/ml to 1µg/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.

2.8.2 Anti-Amastigote Assay

This was carried out as described by Delorenzi et al, (2001). 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 100µl 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, 10ml 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 2000rpm 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) (Berman and Lee, 1984). 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.

2.8.3 Nitric oxide Production Assay

Nitric oxide release in macrophage cultures was measured using the Greiss reaction for nitrites (Hollzmuller et al, 2002). Briefly, 100µl 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, 60µl of Greiss Reagent A (1% sulphanilmide in 1.2M HCl) was added followed by 60µl 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.

2.8.4 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 100μl cells suspension (1×10^6 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 150μl of the highest concentration (1000μg/ml) of each of the test samples at 562nm 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. 10μl 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 (Wang et al, 2006). The results were expressed as the concentration at which the extract inhibited 50% growth of the cells (IC_{50}).

2.9 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. The custom data analysis software program, Chemosen, available at KEMRI was used to determine IC_{50} concentrations.

3. Results

3.1 Yield of plant extracts

The stems parts of *Carissa edulis* were successfully extracted with water, methanol, petroleum ether, dichloromethane and ethyl acetate. The plant extracts obtained were screened for cytotoxicity and in vitro antileishmanial assays. 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. The crude extracts (water and methanol) had higher yields followed by successive extracts (**Table 1**).

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

Plant	% Yield of Extracts (g)					
	Water	**TM**	**PE**	**DCM**	**EA**	**SM**
Carissa edulis	3.66	4.79	0.85	0.33	0.33	1.85

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

Table 2: Phytochemical screening of six solvent extracts of *Carissa edulis*

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

3.2 Phytochemical studies

In this study, the extracts of *Carissa edulis* were screened with the intention of finding out presence or absence of different classes of secondary metabolites in the plant extracts. From the results tabulated in

Table 2, all plant extracts tested positive for more than one phytochemical component. The water extract showed presence of all phytochemical components tested. The dichloromethane extract showed the strongest presence of most phytochemicals, except saponins. The petroleum ether extract also showed strong presence of all phytochemicals, except saponins. The ethyl acetate extract showed weak presence of the phytochemicals tested, with absence of phenols and saponins. Both total and successive methanol extracts showed absence of alkaloids and saponins.

3.3 Bioassays

3.3.1 MIC (minimum inhibitory concentration) anti-promastigote assay

Minimum Inhibitory Concentration (MIC) values for the extracts were determined against *L. major* parasites *in vitro*. These values were also compared to the standard drugs used in the study: Pentostam® and Amphotericin B®. The *C. edulis* petroleum ether extract was the most active with MIC value of 625µg/ml. This was comparable to the standard anti-leishmanial drugs ($p<0.05$). Amphotericin B® showed MIC value of 12.5µg/ml and Pentostam® showed MIC value of 25µg/ml. Both total and successive methanol extracts showed moderate activity with MIC values of 1250µg/ml. The water, dichloromethane and ethyl acetate extracts were the least active with MIC values of 2500µg/ml. Schneider's Insect Medium (SIM) was considered the negative control. The *L. major* parasites continued dividing and proliferating in the media. Thus no anti-leishmanial activity against the growth of parasites was recorded.

3.3.2 Anti-amastigote assay

The plant extracts were screened for their effect on macrophages infected with amastigote forms of *L. major*

in vitro. These were compared with two commercial standard antileishmanial drugs: Pentostam® and Amphotericin B®. RPMI-1640 medium was used as the negative control. All plant extracts showed lower activity comparable to Pentostam® and Amphotericin B® at all concentrations ($p<0.05$). Of interest also, is the effect of these extracts in the reduction of parasites in infected macrophages. The successive methanol extract recorded a multiplication index (MI) value of 11.71% at a concentration of 200µg/ml. It reduced the parasites by 88.29% when compared to the negative control, RPMI ($p<0.05$). This was significantly better than Pentostam®, which recorded a value of 13.16% at a concentration of 200µg/ml ($p<0.05$). The water extract recorded the least parasite reduction activity at a concentration of 50µg/ml. Its MI value was 64.08% when compared to RPMI ($p<0.05$), thus reducing the number of parasites by 35.92% (**Figure 1**).

3.3.3 Nitric oxide Production Assay

Nitric oxide produced by infected macrophages treated with the plant extracts was measured using the Greiss assay. All extracts produced nitrite values significantly lower than Pentostam®, Amphotericin B® and RPMI ($p<0.05$) (**Figure 2**). The water, petroleum ether and successive methanol extracts produced nitrite values of 13.37µM, 12.93µM and 12.82µM respectively at concentration of 200µg/ml. This was comparable to Pentostam® and Amphotericin B® which produced nitrite values of 14.35µM and 14.13µM respectively at concentration of 200µg/ml ($p<0.05$). The ethyl acetate extract produced the least amount of nitrite at concentration of 200µg/ml when compared to Pentostam®, Amphotericin B® and RPMI ($p<0.05$).

3.3.4 Cytotoxicity Assay

Cytotoxicity test of *C. edulis* extracts was performed *in vitro* against vero cells to assess their safety. The ethyl acetate extract was more toxic ($p<0.05$) with an IC_{50} concentration value at 0.65µg/ml than Amphotericin B® ($IC_{50}=0.82$µg/ml). Its petroleum ether extract recorded the least toxic IC_{50} concentration at 3.69µg/ml ($p<0.05$) (**Figure 3**).
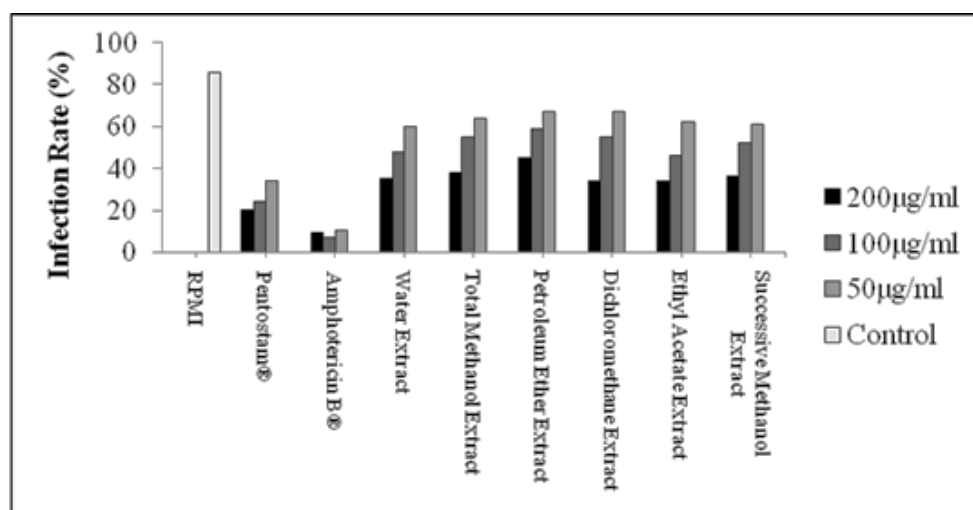

Figure 1: Infection rates of infected BALB/c macrophages treated with six solvent extracts of *Carissa edulis* compared to commercial standard drugs
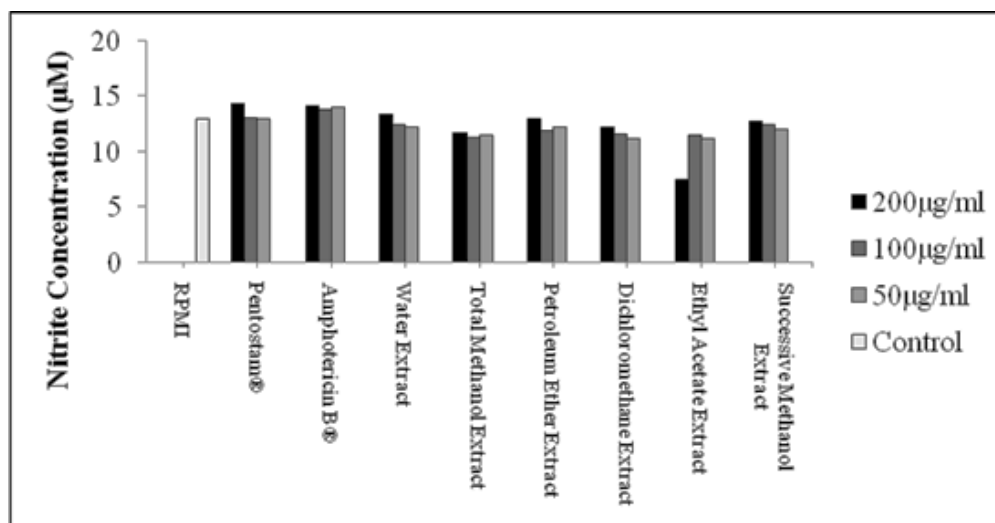
Figure 2: Nitric oxide produced by infected BALB/c macrophages treated with six solvent extracts of *Carissa edulis* compared to commercial standard drugs
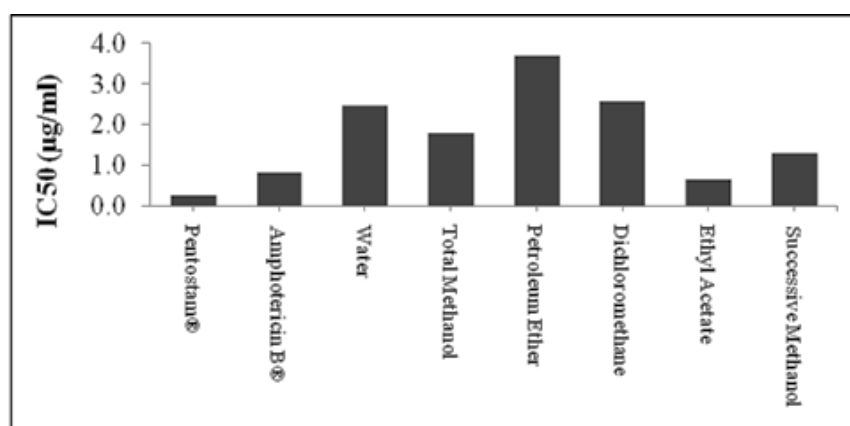

Figure 3: Inhibitory concentration of six solvent extracts of *Carissa edulis* against vero cells compared to commercial standard drugs

4. Discussion

In order to evaluate the efficacy and antileishmanial activity of medicinal plants, different extractions and bioassays are usually carried out (Sofowora, 1993). Solvent extraction was used in this study to prepare extracts due to its ease of use, efficiency and wide applicability (Harborne, 1998). The type of solvent used for extraction 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 (Tonthubthimthong et al, 2001). Preliminary antileishmanial activity screening of plant extracts is usually done using the two forms of the *Leishmania* parasite: the promastigote and amastigote forms. Assays using the promastigote form are easy to perform due to their easy cultivation and maintenance. However, they are seen to only indicate possible antileishmanial activity of the test drug. Thus preliminary screening of drugs using promastigotes needs to be complemented with assays performed using amastigotes (Chan-Bacab and Pena-Rodriguez, 2001). To perform anti-amastigote assays, the promastigote form of the parasite must transform into the amastigote form.

To achieve this, the promastigotes are transformed into amastigotes intracellularly by infecting macrophages of monocytic cell lines derived from mice (Teixeira et al, 2002).

In this study, preliminary antileishmanial screening of *Carissa edulis* extracts performed on the promastigote form of *Leishmania major* showed that their activity against promastigotes is not in relation to their polarity. The less polar petroleum ether, polar total methanol and successive methanol extracts recorded moderate activity while the water and less polar extracts (dichloromethane and ethyl acetate) recorded weak activity. The activity of these extracts against the amastigote form of *L. major* was seen to be dose-dependent. Higher concentrations of *C. edulis* extracts seemed to reduce the infection rate of macrophages. The polar extracts (water, total methanol, successive methanol and ethyl acetate) were found to be more active than less polar extracts (petroleum ether and dichloromethane). At their highest concentrations (200µg/ml) all extracts, except petroleum ether, had IR values not significantly different to the standard reference drug Pentostam at concentration 50µg/ml ($p > 0.05$). This trend though did not translate to their

multiplication indices. The successive methanol extract at a concentration of 200µg/ml reduced the number of amastigotes by 88.29% effectively than Pentostam at the same concentration (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 (James, 1995). The petroleum ether and crude (total) methanol extracts reduced the number of amastigotes also demonstrated good potential activity against *L. major* amastigotes compared to Pentostam and Amphotericin B® (p<0.05). The ethyl acetate, water and dichloromethane extracts recorded the least MI values.

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 (Sternberg et al, 1994; David et al, 1997; MacMicking, Xie and Nathan, 1997; Mosser, 2003). Its production as an important step in antileishmanial activity in murine macrophages has been documented (Augusto et al, 1996; Leiw et al, 1997; Ellis et al, 1998; Louis et al, 1998; Kavoosi et al, 2006). In this study, infected macrophages treated with *C. edulis* water extract at a concentration of 200µg/ml produced 3% (13.37µM) more NO than non-treated macrophages containing RPMI-1640 medium (12.98µM) as the control. The extract showed good antileishmanial activity against amastigotes with an infection rate, reducing the number of amastigotes by half. This data proposes that the phenols detected in the water extract may influence the production of nitric oxide by infected macrophages. This action activates the killing mechanism of NO against the *L. major* parasites. A study by Mendonça-Filho et al, (2004) reported that phenols present in the husk fiber of C*ocos nucifera* L. (Arecales: Arecaceae) induced the production of nitric oxide by murine macrophages. Although the successive methanol extract showed good potential antileishmanial activity against promastigotes and amastigotes, the extract did not produce increased levels of NO compared to the control (p<0.05). This suggests that the mechanism of action of *C. edulis* successive methanol extract does not involve nitric oxide. Its action could involve either killing the parasites directly or inhibiting the parasite growth due to specific compounds present in the extract. TLC visualization reagents aided in screening for the presence of active compounds present in *C. edulis* extracts. According to the results, one or more compounds were present in the extracts which attributed to their antileishmanial activity either synergistically or individually. Petroleum ether and dichloromethane extracts indicated strong presence of terpenoids. Terpenoids are soluble in solvents with low polarity (Dzubak et al, 2006). A study done by Ogungbe et al, (2013) reported antileishmanial activity of terpenoids against *L. donovani* and *L. tropica*. Another study done by Tan et al, (2002) reported antileishmanial activity of terpenoids against *L. donovani* and *L. major*. The water, petroleum ether and dichloromethane extracts showed strong presence of phenols. Although antileishmanial activity of phenols has been reported (Chen et al, 1994; Koide et al, 2002; Oliver et al, 2003;

Bodiwala et al, 2007), the water and dichloromethane extracts showed weak activity against *L. major* promastigotes. This therefore suggests that the phytochemicals did not influence their activity towards the parasites. However, these extracts showed good activity against *L. major* amastigotes. This could be due to the strong presence of alkaloids in the dichloromethane extract, and presence of saponins in the water extract. Antileishmanial activity of alkaloids has been reported (Fournet et al, 1993; Fournet et al, 1994; Fournet et al, 1996; Henriques et al, 2001; Ferreira et al, 2002; Assmar et al, 2003). There have also been reports on antileishmanial activity of saponins (Delmas et al, 2000; Maes et al, 2004a; Maes et al, 2004b; Mori et al, 2007).

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 *C. edulis* 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 (Berridge et al, 2005). A progressive decrease in cytotoxicity of extracts of *C. edulis* was observed with decreasing polarity. The petroleum ether, dichloromethane, water, total methanol and successive methanol extracts showed no cytotoxicity towards growth of vero cells. These extracts also showed good potential antileishmanial activity against *L. major* parasites. Therefore, this suggests that they are safe to use. The ethyl acetate extract was however toxic to vero cells (IC_{50}=0.65µg/ml) despite its potential antileishmanial activity against the parasites. Its cytotoxicity could be due to presence of terpenoids in the extract, which are associated with their cytostatic property (Dzubak et al, 2006).

5. Conclusion

The results of this study revealed the antileishmanial activity of *Carissa edulis*. The crude (total) and successive methanol extracts showed potential activity against *Leishmania major* promastigotes and amastigotes with no toxicity reported in mammalian (vero) cells. The water and dichloromethane extracts showed potential activity against *L. major* amastigotes with no toxicity reports. The petroleum ether extract showed potential activity against *L. major* promastigotes with no toxicity reported. The ethyl acetate extract showed potential activity against *L. major* amastigotes, though it was toxic to vero cells. The phytochemical screening results also shed light on the activity portrayed by these extracts. Therefore all *C. edulis* extracts showed potential antileishmanial activity against *L. major* parasites.

Conflict of Interest

The authors declare no conflict of interest.

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