In vitro inhibitory effect of *Ambrosia maritima (Asteraceae)* extract on the survival of *Leishmania donovani*

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Abstract: The *in vitro* susceptibilities of the isolates strain of *Leishmania donovani* (EMA, EAO, ZAA, HIH, MYIO and HYEA) to Sodium stibogluconate (SSG) and the experimental crude extract of *Ambrosia maritime* L. *(Asteraceae*) were determined for extracellular log-phase promastigotes and interacellular amastigotes in peripheral blood mononuclear cells (PBMCs). Susceptibility to reference drug (Pentostam) with different concentrations (1000, 500, 250, 125, 62.5 and 31.25) showed an increase in growth inhibition with the reduction in the concentration till then decrease in growth inhibition with the more reduction in concentration. While the *Ambrosia maritima* crude extract showed the decrease of growth inhibition with the increasing in concentration after 24, 48 and 72 hours. Ethanolic crude extract of *A. maritime*, it caused growth inhibition of *L. donovani* promastigotes at (1000, 500, 250, 125, 61.5, 31.25 µg/ mL), but the concentration 500µl/ml of the extract showed high biological activity 100% the study indicated that further analysis still to be done on the active crudes; bio-guided fractionation should also be conducted and may lead to the isolation of the major components in the active crude. Higher potency was observed against intercellular amastigots stage (IC_{50} is different for all parasites isolates EMA=1.339 to.541 EAO=1.473 to1.719, ZAA=1.447 to 1.719, HIH=1.465 to 1.686, MYIO= 1.280 to 1.559 and HYEA=1.473 to 1.707 µg/mL), a similar was observed for reference drug (SSG) (IC_{50} =1.945 to2.788µg/mL).

Key words: *Ambrosia maritime, Leishmania donovani*, MTT assay

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Introduction

Leishmaniasis is a group of diseases caused by protozoan parasites,over 20 pathogenic species of the *Leishmania* parasite are known that are transmitted to humans by the bite of infected female *phlebotomin*e sand flies. Leishmaniasis occurs in three clinical forms: (i) cutaneous leishmaniasis (CL), which is caused by *L.major, L. tropica, L. aethiopica, L.infantun, L. chagasi* (Mediterranean and Caspian Sea region CL), *L. amazonensis, L. mexicana, L. braziliensis, L. panamesis, and L. peruviana*; (ii) mucocutaneous leishmaniasis (MCL) or espundia, which is caused by *L. brazilensis, L. panamensis, L. guyanensisin L. infantum and L. donovani*; (iii) visceral leishmaniasis (VL), which is caused by species of the *L. donovani* complex that consist mainly of *L. infantum, L. donovani, and L. chagasi*. VL is also known as kala-azar, black fever, and Dumdum fever. There is a fourth form known as diffuse cutaneous leishmaniasis (DCL), which is caused by *L. amazonensis* and *L. aethiopica*. (Elmahallawy *et al*., 2014) The parasite isolated from humans and sand flies that causes VL belongs to the *Leishmania donovani* sensual to cluster. There are few reports of isolation of *Leishmania archbaldi* and *Leishmania infantum* from humans and dogs in Gedaref State, eastern Sudan. (Zijlstra *et al.,* 2001).Post-kala-azar dermal leishmaniasis (PKDL) as mentioned above, the species of *Leishmania* strongly determines how the disease will evolve. *Phlebotomus orientalis* is the primary vector for transmission of VL in Sudan. (Federal Ministry of Health, Republic of Sudan, NTDs 2014). Visceral leishmaniasis (VL) or 'kala-azar ', responsible for an estimated 200,000 to 400,000 infections each year worldwide (WHO, 2010).WHO estimated that 90% of global VL cases occurred in 6 countries: Bangla-desh, Brazil, Ethiopia, India, South Sudan and Sudan. Of the global number of cutaneous leishmaniasis (CL) cases, >70% occurred in 10 countries: Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, the Islamic Republic of Iran, Peru, Sudan and the Syrian Arab Republic (WHO 2016).

In Sudan Visceral leishmaniasis is one of the neglected infectious diseases, Sudan ranks third among the VL high-burden countries after India and South Sudan, which together with Bangladesh, Brazil and Ethiopia account for 90% of VL cases worldwide, according to recent WHO estimates (WHO 2016). The highest fatality rate was observed in the year 2002 (4.8%); this rate had declined in 2014 (1.1%) and 2015 (1.7%), most of this rate are children 22 590 children per year 201643.6% (Adama *et al.* 2016).

The VL control strategy is based on early diagnosis and treatment, vector control, health education and operational research. Usually, the first approach for laboratory diagnosis of cases suspected of VL in Sudan is serology, using either the direct agglutination test (DAT) or rK39-based rapid diagnostic tests (RDT). The second approach is lymph node aspirate microscopy. Bone marrow and splenic aspiration, however, are rarely performed in Sudan due to technical and logistical difficulties and are limited to reference laboratories (WHO 2015) (Zijlstra *et al*. 2001). In recent years, molecular tests such as the polymerase chain reaction (PCR) and quantitative real-time PCR, when performed on peripheral blood, have shown high sensitivity and specificity in the diagnosis of VL and treatment monitoring (Antinori *et al* 2007) (Salam *et al* 2010).

Chemotherapy of parasitic diseases including leishmaniasis is still challenging, the drug efficacy is mostly limited by the inability of the pharmaceuticals to reach its target in a sufficient concentration and for a sufficient duration. The available drugs currently in use for the treatment of leishmaniasis include the followings:

First-line treatment comprises combination of sodium stibogluconate (SSG; Albert David, Kolkata, India) and paromomycin sulphate (PM; Gland Pharma, Hyderabad, India) (Hamad *et al*., 2010), (Ahmed Musa; *et.al* 2012).Second-line treatment of visceral leishmaniasis Liposomal amphotericin B *(*AmBisome) is administered at a dose of 3mg/kg/daily days for 10 to 14 days. 3-5 mg/kg per dose for 6-10 days up to total of 30 mg/kg. Miltefosine (hexadecylphos- phocholine, HePC),has demonstrated activity against both leishmania parasites and human cancer cells, linking its activity mainly to (i) apoptosis and (ii) disturbance of lipid-dependent cell signalling pathways , cytochrome-c oxidase was inhibited. (Luque-Ortega *et al.* 2007).

Victims of leishmaniasis are generally poor, lengthy treatment using expensive drugs with related costs is far beyond the means of such families (Oryan *et al*., 2014). Given the above mentioned reasons, development of new, less toxic and cost-effective drugs with greater efficacy as well as more accessible alternative therapeutic strategies which could become available for low income populations to treat the disease has become a necessity. Some researchers have been looking for new alternatives in nature as an important source of drugs used in medicine (Gamboa- Leon *et al*. 2014; Sifaoui *et al*. 2014). It has been estimated that there are about 250,000 medicinal plant species in the world. The present study aims to investigate the *in vitro* inhibitory effect of some local plants of medical value on *Leishmania donovani*. *Ambrosia maritima* L. *(Asteraceae*) is a widely distributed plant in African

countries, the species was found in Sudan and Egypt. In Sudan, it grows in Nile bank; it belongs to the subfamily *Tubuliflora*, which is a branch of the family *Compositae* /Astersces of flowering plants. Traditionally, it is used to cure gastrointestinal disturbance, abdominal pain, kidney inflammation and renal colic. In addition, their therapeutic properties extend to include anti-molluscicidal, anti-malarial and anti-tumor activities. (Abdelgaleil *et al.* 2011; Khalid *et al.* 2012). Promising candidates to treat refractory tumors. (Mohamed *et al*. 2015) and anti diabetic (Eman 2014). Drinking decoctions of Damsissa were the most commonly used remedy for schistosomiasis in Upper Egypt (Abdel-Hamid and Tarabanko, 2004). The phytochemical investigation of *A. maritima* revealed the presence of pseudoguaianolide sesquiterpene lactones ambrosin, damsin, damsinic acid, neoambrosin, hymenin [7, 6, and 3] and 11β-hydroxy-1-chloro-11, 1-dihydrohymenin (Ali Makkawi, .2015).

MATERIAL AND METHODS:

Ethical consideration:

The study was ethically approved by the Ethics Committee of the Institute of Endemic Diseases, University of Khartoum.

Parasites:

Leishmania donovani parasites were isolated from Visceral leishmaniasis (VL) patients who attend El-Hassan Center for Infectious Diseases in Dokka, Gedarif State. Prior to parasite isolation patients were tested with a direct agglutination test to confirm the infection.

Parasite isolation and cultivation:

Lymph node and bone marrow aspirations were carried out to isolate *Leishmania donovani* parasites. Aspirates were inoculated into tubes containing NNN medium supplemented with antibiotics (penicillin/streptomycin 1 %.) and 10% Fetal Calf Serum (FCS). Culture tubes were then incubated at 25 C^{o} for 2 weeks. Promastigotes at stationary phase were frozen at -196^{o}C until used.

Leishmania DNA extraction:

According to described modified method to (Kemp, M., *et al* 1991) Promastigotes were recovered, pelleted and properly mixed with 1 volume of guanidine-HCL solution in a test

tube. The ratio sample: guanidine was increased to 1:6, and then the mixture was boiled for 15 minute in water path after that the mixtue was stored at -20^{o}C in dark. For DNA extraction the mixture was boiled again for 15 minute and 10µl of lysis buffer was added before incubation at room temperature over night. Then 5 ml guanidine (for each sample), 300 µl NH_4 acetate and 10 µl protinase K were added and the sample were then vortexed for few second and the tubes were incubated at 37^{o}C (overnight). 2 ml of cold chloroform was added and centrifugation was performed at 6000 rpm for 10 min. The clear layers were transferred into new tubes for each sample after that the cold absolute alcohol was added complete to 10 ml to each sample then aliquot in 15 ml tissue culture tubes. Centrifuged at 6000 rpm for 20 minutes. Discarded alcohol and drying the DNA stained with ethedium bromide and run in agarose gel and visualized in UV illuminator. Genomic DNA (515 ng) in 10 µl of ddH_2O was used for amplification of Leishmania genomic DNA.

Amplification of Leishmania DNA

Clinical isolates were characterized based on the size of their minicircle DNA as previously described (Ibrahim ME. *et al.*, 2001). 10 ng of parasite DNA was amplified using two primers (AJS3 and DBY). The primer position is on the conserved region of the minicircle DNA and encompassing part of the sequence of origin of replication. In amplification, these primers yielded a whole length minicircle sequence, which differs between the different Leishmania species. In this PCR procedure The DNA samples were thawed and vortexed. The reaction volume was 30µl per sample and performed in a 0.2 ml thin walled micro-centrifuge tubes. The mixture contained 5µl of 10X reaction buffer (Promega, Madison WI, USA) with a final concentration of 1X, 2µl of 20 mMdNTPs mixture (0.2 mM of dTTP, 0.2 mMdATP, 0.2 mM dCTP & 0.2 mM dGTP), 3µl of 25 mM $MgCl_2$ (Promega, Madison WI USA), 2.0µl of primers mixture (forward: 5' GGCATAAATCCATG TAAGA 3'; reverse: 5' TGGCTTTTATATTATCATTTT 3'), and 0.25µl of thermo-stable Deoxyribonucleic acid (DNA) polymerase (Promega, Madison WI, USA) (5U/µl) to each PCR tube, 5µl of Deoxyribonucleic acid (DNA) templates were added, and the PCR mixture was completed to 30µl with double distilled water. The following programme was fed to the PCR machine and was run for 35 cycles: initial denaturation at 94^{o} C for 3 minutes, annealing at 64^{o}C for 1 minute, extension at 72^{o}C for 1 minute and denaturation at 94^{o}C for 30 seconds. A final extension cycle at 72^{o}C for 10 minutes was included. The amplification reactions were

analyzed by agarose gel electrophoresis, followed by ethidium bromide staining and visualization under UV light.

Plant collection

Ambrosia maritima L., *Asteraceae* leaves were collected during January-February 2017 from University of Khartoum farm in Shambat and from the banks of river Nile around Khartoum. Plants were identified and authenticated by the taxonomist Yahya Sulieman Mohamed in Herbarium of Medicinal and Aromatic Plants and Traditional Medicine Research Institute (MAPTMRI), National Centre for Research, Khartoum, Sudan. Demsissa (local name) materials collected were washed under running tap water to remove soil particles and other dirt, air-dried in the room temperature (25^{o}C) for many days. The dried samples were grounded with a mixer grinder. The powder was stored in bottles at room temperature before extraction.

Preparation of crude extracts:

100g of each plant (leaves of *Ambrosia maritima* were extracted with 80% ethanol (200 ml) using Soxhlet apparatus (boiling point50^{o}C) for 8 hours. Extracts were filtered and ethanol was evaporated at room temperature for several days until complete drying. Each residue was weighed and the yield percentage was calculated and then stored at- 4°C in a tightly sealed glass vial for further use.

Phytochemical Screening of Active principles of the plants:

The tested plant was subjected to the phytochemical analysis as explained by the crude extract as follows; tests for carbohydrates and/or glycosides (Molish`s test, Fehling's test and Benedict's test), test for tannins, test for alkaloids, and flavonoids as described by Smolenski *et al*., (1974), test for saponins and flavonoids, testes for tri- terpenes as described by Dafert *et al*., (1934).

Preparation of extract, Solutions:

Using sensitive balance 100 mg of crude extraction was weighed and in 50 ml tissue culture tubes and dissolved in 100 ml M199 medium by 5-min sonication to make a concentration of 1000 µg/ml Stock (S) and passed through 0.22µm filter then stored at – 20 ^{o}C for use .

Serial dilution of plant extracts stocked and control were prepared. Fixed amount of stock (S) was taken and added the same amount of M199 medium in equal volume to make (500, 250,125, 62.6 etc) µg/ml. The mixture was vortexed and stirred to obtain a homogenous solution. Also, serial dilution of reference drug was prepared.

Drug:

Sodium stibogluconate B.P. (Pentostam—equivalent to 100 mg pentavalent antimony/ml) manufactured by ALBERT DAVID LIMITED 15 Chittaranjian Aveneue Kalkata 700 072 at 5/ 11 D Gupta lane, Kolkata 700 050, INDIA was used as a reference drug in this study.

Cell Line and Cytotoxicity screening:

Cytotoxicity screening of *Ambrosia maritima* was evaluated by colorimetric cell viability MTT assay using the method described by (Mahmoudvand *et al.*, 2014). L20B (Normal monkey cell line) was obtained from MAPRI, were maintained in a flask containing MEM (minimal essential medium) and supplemented with 10% FBS and 90% minimal essential medium (MEM) and then incubated at 37^{o}C and 5% CO_2. The cells were subcultured twice a week. Cell suspension was counted and adjusted to 2.5 x 10^5 /ml.

Cell counting:

Cells were counted using the improved Neubauer chamber. The coverslip and chamber were cleaned with detergent, rinsed thoroughly with distilled water and swapped with 70% ethanol, then dried. An aliquot of cell suspension was mixed with an equal volume of 0.4% trypan blue in a small tube. The chamber was charged with cell suspension. After cells had settled, the chamber was placed under a light microscope. Using 40 X objective, cells in the 4 large corner squares (each containing 16 small squares) were counted. The following formula was used for calculating the cells:

$$(\text{Cells/ml})\text{N} = \frac{\text{Number of cells counted} \times \text{Dilution factor} * \times 104}{4}$$

*Dilution factor is usually 2 (1:1 dilution with trypan blue), but may need to further dilute (or concentrate) cell suspensions.

MTT assay:

Serial dilutions of extract were prepared in a 96 well flat bottomed plate (Nalge Nunc, Inter.). The outer walls of the plate were filled with 250 µl of in-complete culture medium except the last row 6 middle wells (B - G), which were used for the negative control receiving 50 µl of culture medium and 2µl of sterile 0.5% Triton X. 50 µl/wells complete culture medium (CCM) were added and 30 µl more were added to second column wells (B – G) that were used as first extract dilution wells. To the first dilution wells in the row, 500 µg of c

suspension extract were added to the 80 µl. The extracts were then serially diluted by two-fold dilution from well B3 till B11 by transferring 250 µl to the next well after proper mixing. From the last dilution wells (B-11), 50 µl were discarded. Each compound was tested in triplicate. Cell suspension in a complete culture medium containing 2.5 X 10^5 /ml was properly mixed, and 150 µl of it were transferred into each well of the plate. The plate was covered and placed in 5% CO_2 incubator at 37 °C for three-five days (72 hours-120 hours). On the third/fifth day, the supernatant was removed from each well without detaching cells. MTT ((3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole) stock (5 mg/ml) was prepared earlier in 100 ml PBS (phosphate buffer saline). MTT suspension was vortexed and kept on a magnetic stirrer until all MTT dissolved. The clear suspension was filter sterilized with 0.2 µ Millipore filter and stored at 4 °C or – 20 until use. MTT was diluted (1:3.5) in a culture medium and brought to room temperature. To each well of the 96 well plates, 50 µl of diluted MTT were added. The plate was incubated further at 37° C for 2 to 3 hours in CO_2 incubator. MTT was removed carefully without detaching cells, and 200 µl of DMSO were added to each well. The plate was agitated at room temperature for 15 minutes then read at 540 nm using micro plate reader**.** The percentage growth inhibition was calculated using the formula below:

% cell inhibition = 100-{(Ac-At)/Ac} × 100

Where, **At** = Absorbance value of test compound; **Ac** =Absorbance value of control.

***In vitro* susceptibility of promastigotes to plant extracts:**

The in vitro screening assays of promastigotes were done according to (Ibrahim *et a*l., 1994). *L. donovani* promastigotes were harvested at day 6 of culture and the count was adjusted to 2×10^7 parasites/ml. The stock plant ethanol extracts (100 mg/ml) were diluted with culture medium for working concentrations (1000, 500, 250, 125, 62. 5, 31.25µg/ml). A volume of 500 µl from each parasite culture was transferred into 48-well flat bottom microtiter plate and incubated at 26^{o}C for 1 hour. Serial concentrations of each extract were added to the appropriate cells in the plate and the parasite/extract suspension was gently mixed. And the next concentration was added to second well of each row. Positive control wells (drug) contained only promastigotes (2.7X 10^6 parasites /ml). Serial dilutions of the drug was added accordingly as in the extracts wells. Normal control wells contained only medium. The plates

were incubated at 26° C and promastigotes were counted after 24, 48, and 72 hours using haemocytometer. (Protocol 5.0.1 DNDi-LEAP Protocols Handbook Reviewed 2010).

$$\% \text{ Inhibition} = \frac{\text{Mean No.of live parasites} - \text{Mean BG} \times 100}{\text{Mean No.of control}}$$

BG: background wells containing medium only.

Screening of amastigotes sensitivity:

Separation of peripheral blood mononuclear cells (PBMCs):

This modified assay performed according to (Kemp *et al.,* 1991). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood of healthy adult volunteer using density gradient centrifugation with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). 5 ml of venous blood was collected in the heparinised blood collection tube. 5 ml of Ficoll-hypaque were added to 15 ml tube, carefully overlaid an equal amount of blood and centrifuged at 1400 layer rpm for 25 minutes without brake. Carefully aspirated the mononuclear dense ring into sterile 15 ml centrifuge tubes with 1 ml fresh RPMI-1640. Washed the collected cells 2 times in 10 ml RPMI-1640.Then, centrifuged at 1200 rpm for 10 minutes after each wash, suspend cells pellet in 2 ml RPMI-1640media enriched with 10% heat-inactivated fetal calf serum (FCS).

Counting of peripheral blood mononuclear cells (PBMCs):

Cell pellets were diluted (suspension in complete culture media) 1 in 2 times trypan blue stain. The number of viable cells/ml was counted carefully by pipetting 50 ul of diluted cells into a haemocytometer under the 40x objective lens of the compound microscope.

Culture of (PBMCs):

Killing of intracellular *L.donvani* amastigotes was assayed by analyses of the number of infected cells in macrophage monolayers. Freshly isolated human monocytes were plated in 24 well flat-bottomed cell culture plates, at a density of 1×10^6 cells per well in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin 1%. After 24 h of incubation at 37C in 5% CO_2/95% air atmosphere for 24 hours, Promastigotes of each isolate (in a ratio of 5 parasites:1 macrophage) were added to each well and were incubated at 37°C in a CO2 incubator (5% CO2 and 95% relative humidity). After 48 hours free parasites were removed by washing with 200uL of warm RPMI-1640 medium per well (very careful), Aspirate the medium and add 100uL of fresh complete medium, and infected macrophages were treated

with different concentrations of plant extracts.2uL of each compound stock solution (31,25 µg/ml) was added into15 ml centrifuge tubes, pipette up and down to mix and then transfer 1 ml to the next tube in order to make serial dilutions(15.6 ,7.8, 3.9,1.9,0.9 ug/ml). Then the different concentrations were added to each well (Pipette carefully to not detach the macrophages). Then added 100uL of fresh medium and incubated for 72 h at 37°C, 5% CO_2 and 95% relative humidity. Finally, dried plate wells were fixed with methanol (99.9%), stained with Giemsa (10%) for 10 minutes and studied under a microscope. In this step, macrophages containing *leishmania donovani* amastigotes without treatment (negative controls) and macrophages with *leishmania donovani* amastigotes and pentostam treatment were considered as positive and macrophages containing *leishmania donovani* amastigotes with plant extract treatment (Mahmoudv *et al*. 2014).

Antileishmanial activity of extracts was evaluated by the numbers of amastigotes were determined in cultures. The results were expressed in terms of infection rate (IR) and the multiplication index (MI) (Ogeto *et al.* 2013 and Ray, Dittel 2010) was calculated as follow:

IR = Number of infected macrophages in 100 macrophages

$$MI = \frac{\text{Number of amstigotes in test culture /100macrophages}}{\text{Number of amastigotes in control /100 macrophages}} X100$$

In addition, IC_{50}s values were calculated by using the values for the number of amastigotes/macrophage (calculated by Prism analysis).

Statistical analysis

Prism Software Graph Pad Prism 8.1.2 (Graph Pad Prism version 8.1.2 software Inc., San Diego, CA)) was used for data entry and statistical analysis and the differences between samples were determined by using one way analysis of variance (ANOVA) test. Moreover, to compare the IC_{50} values of plant extracts and control drug *t*-test performed. *P*-value of less than 0.05 was considered statistically significant.

Results:

Molecular characterization of the isolates:

Leishmania donovani field isolates were subjected to DNA amplification using species-specific primers that amplify a whole length minicircle gene. DNAs yielded bands of a molecular weight approximately 540 bp, which corresponds to the *L.donovani* complex

minicircle size, thus confirming the identity of the parasites analyzed (Fig. 1). The result shows that all parasites DNA had molecular weight of 540 bp.

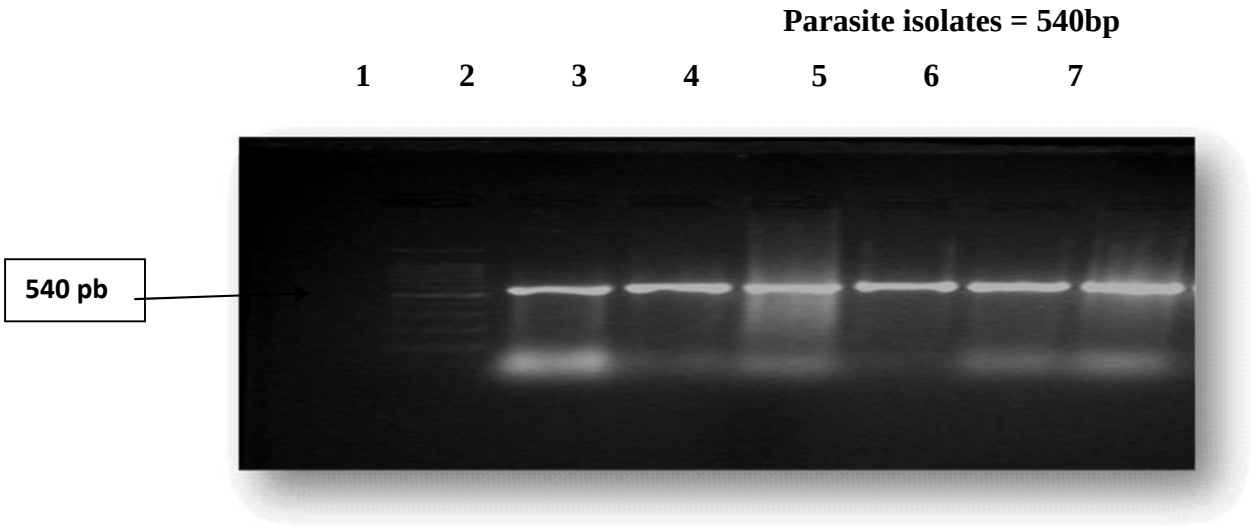

Figure (1). PCR result of field isolates using species-specific minicircle primers. The PCR product size is approximately 540 bp. Lane 1: 1kb marker (ladder); Lanes 2-7: field isolates from East Sudan.

Plant extracts analysis:7

Qualitative phytochemical analysis of the extract showed that *Ambrosia maritima L. (Asteraceae*) extract contains, saponins, tannins, amino acids, carbohydeates and a lot amount of tri- terpens. Flavonoids and alkaloids were found.

Cytotoxicity screening:

The colorimetric cell viability MTT assay used to evaluate the cytotoxicity the plant extracts showed it has no toxic effect on the mammalians cells (L20B, Normal monkey cell line) (**Table 3.2**).

***In vitro* susceptibility of promastigotes to plant extracts:**

In vitro effect on *Leishmania donovani* promastigotes. The sensitivity of the *L .donovani* promastigotes to SSG and crude ethanolic extracts of plant *Ambrosia maritima L. (Asteraceae*) and is shown in (Figures (2 and 3). The crude ethanolic extracts of plant *Ambrosia maritime* showed a decrease in growth inhibition on the promastigotes isolated from VL patients who were non responsive to SSG treatment. The results showed were a mean of experiments carried out at 24, 48 and 72 hours. Increasing concentrations of plant extracts inhibited the growth of the parasite in linear fashion compared to the control values in both sensitive and resistant isolates.The concentration 500µl/ml of *Ambrosia maritima*

Figures (3). Showed their full inhibition of the promastigotes as seen at the same concentration of the pentostam Figures (2).

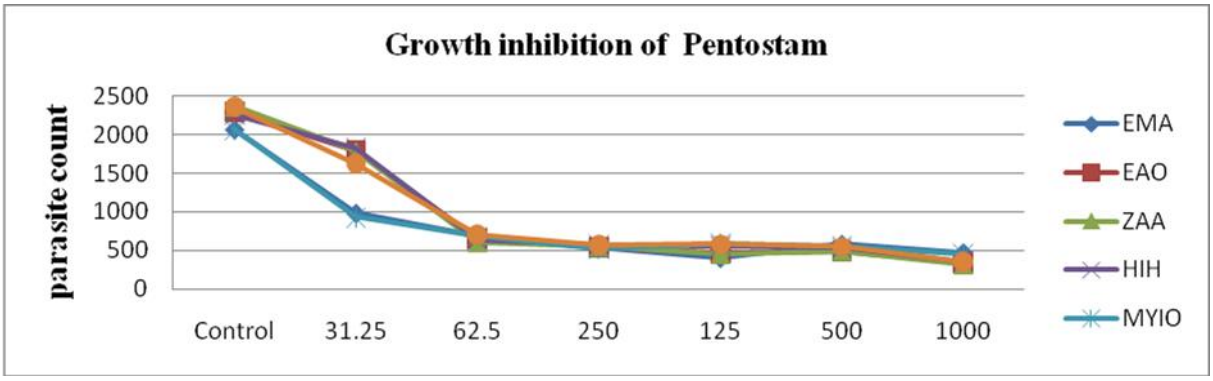

Figure (2) The mean growth inhibition of promastigotes after 72 hours. *In vitro* using **Pentostam** at different concentrations against different isolates of *L.donovani* from VL patients non-responsive to SSG treatment.

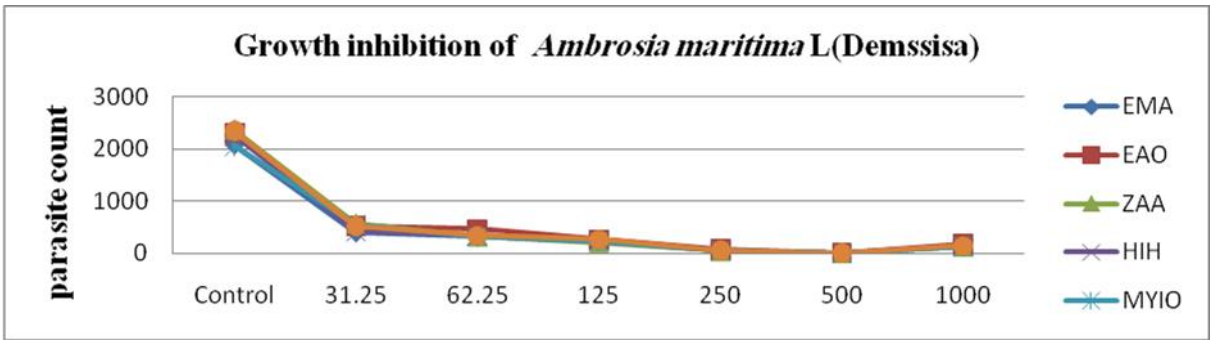

Figure (3). Show the mean growth inhibition of promastigotes after 72 hours. *In vitro* using ***Ambrosia maritima* L (Demssisa)** at different concentrations against different isolates of *L. donovani* isolated from VL patients non-responsive to SSG treatment.

***In-vitro* screening of amastiogte sensitivity**:

In vitro studies on activity of ***A. maritime* (Demssisa)** and ***C. schenanthus (Maharaib)*** against amastigotes of ***L. donovani*** was screened. The extract of two plants showed potent activity with no effect on mammalian cells. The activity of extracts evaluated by counting of number of amastigotes in each macrophage by examining 100 macrophages in comparison to positive control (pentostam) and untreated macrophages. Based on the results presented on

figures (4 and 5) were used to determine the infection rate, multiplication index and IC_{50} using pentostam as positive control.

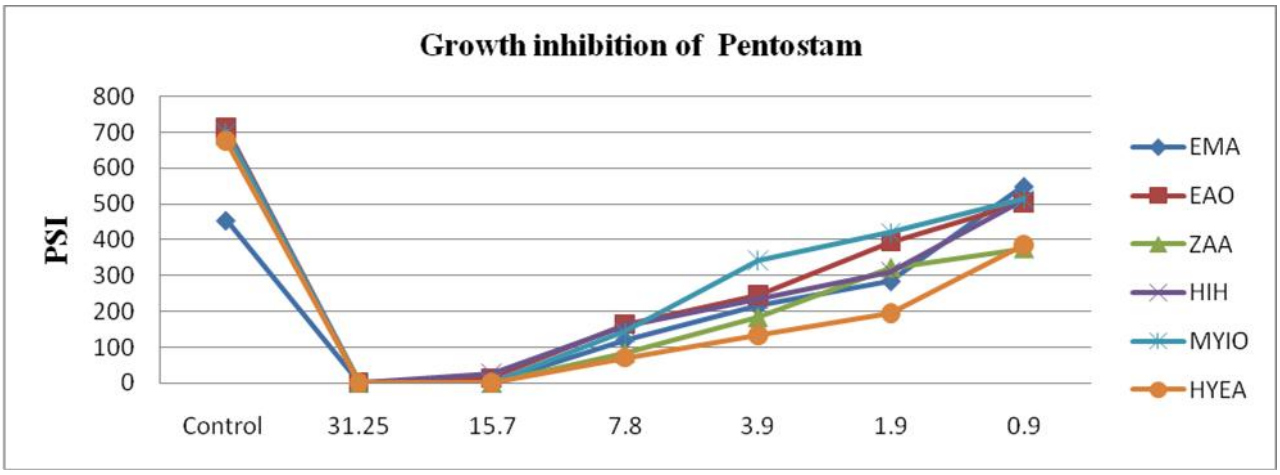

Figure (4) Effect of pentostam on the survival of six *L. donovan*i amastigotes using macrophage system.

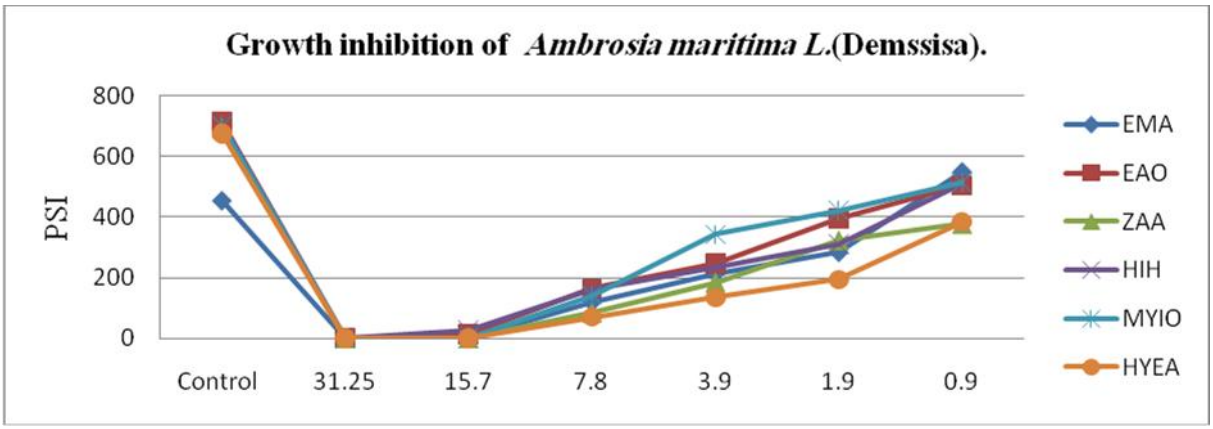

Figure (5) Effect of ***Ambrosia maritima*** **L (Demssisa)** on the survival of six *L. donovan*i amastigotes using macrophage system.

DISCUSSION

Natural products are potential sources of new and selective agents for the treatment of important tropical diseases caused by protozoans and others diseases (Ezatpour B *et al*., 2015)(Radtke OA, *et al*., 2003) Over 100 plants have been reported to be active against various forms of leishmanial parasites (Rocha *et al*., 2005). In vitro screenings of various medicinal plants currently used in traditional medicine are essential and important first steps to prove the efficacy and safety of these plants in the treatment of infectious diseases The studies showed that the *Ixora coccinea* leaves extract having anti leishmanial activity against the promastigotes of *L. donovani* (Naskar et al., 2013). The root extract of *Perovskia abrotanoides* shows anti leishmanial activities against the *L. major* (Jaafari et al., 2007). The pharmacological screening of methanolic extract of *Aloe vera* leaves and *Tamarix aphylla* bark were assessed to investigate the *in vitro* anti leishmanial activity of the medicinal plants against cutaneous leishmaniasis by (Iqbal *et al*., 2012).

To conduct this *invitro* study, firstly it was confirmed that field isolated samples returned to *leishmania donovani* DNAs yielded bands of a molecular weight approximately 540 bp, which corresponds to the *L.donovani* complex minicircle size, thus confirming the identity of the parasites analyzed (Fig. 1). The result shows that all parasites DNA had molecular weight of 540 bp. While the phytochemical analysis of ethanolic crude extracts of *A. maritima* showed that *Ambrosia maritima L. (Asteraceae*) extract contains, saponins, tannins, amino acids, carbohydeates and a lot amount of tri- terpens. Flavonoids and alkaloids were found. Also, the colorimetric cell viability MTT assay used to evaluate the cytotoxicity the extracts of *A. maritima* and according to protocol followed showed it has no toxic effect on the mammalians cells (L20B, Normal monkey cell line) with IC_{50} = 567.2454 µg/ml (**Table 2**).This present study designed to obtain preliminary results on the anti leishmanial effects of some selected Sudanese medicinal plant *A. maritima.* The plants were considered for investigation on the basis of it traditional use for local and systemic condition of leishmaniasis and based on similarity in the chemical composition with plants belonging to the same genera that have anti-leishmanial activity. The plants that showed activity against *Leishmania donovani* promastigotes are edible and traditionally used as medicinal plants for the treatment of various illnesses. Plant extracts were subjected to antileishmanial activity tests using the promastigote and amastigote stages of *L. donovani.* The study showed that pentostam increase in growth inhibition with the reduction in the concentration till then decreases in growth inhibition with the more reduction in concentration. Other agent showed

the decrease of growth inhibition with the increasing in concentration after 24, 48 and 72 hours (figure 2). While ethanolic crude extracts of *A. maritima L.* it caused growth inhibition of *L. donovani* promastigotes at (1000, 500, 250, 125, 61.5, 31.25 µg/mL), but the concentration (500µl/ml) of *Ambrosia maritima L.* showed100% biological activity (figure 3) where compared with reference drug (Pentostam). The anti-leishmanial activities of these secondary metabolites were reported from other plant species (Shah NA, *et a*l., 2014), a number of studies have demonstrated that many plant extracts exhibit activity against *L.donovani* this studies found the same result of plants from the same family, crude extract from the shoots of *Artemisia inculta* Delile and *Carthamus tinctorius* L. with activity of 84.1%, IC_{50} = 8.8 µg/mL, 82.1%, IC_{50} = 37.01± 0.001(Omar Hamarsheh *et al*. 2017). Also the other species showed the activity, Shoots of *Artemisia inculta* and *Malva sylvestris* with activity of 84.1%, IC_{50} = 8.8 µg/mL. And 90.1%, IC_{50} = 19.5 µg/mL respectively (Omar Hamarsheh *et.al* (2017).On the other hand the samples were assayed on intracellular amastigote stage of the test isolates, these plant extracts. at concentration. (31.25, 15.6 , 7.8, 3.9, 1.9, 0.9 ug/ml) showed activity against *L .donovani* amastigotes The activity of extracts evaluated by counting of number of amastigotes in each macrophage by examining 100 macrophages the highest concentration(31.25 ug/ml) in pentostam and *Ambrosia maritima* showed the high activity **(figure(3.5 and 3.6) .**The study indicated that further analysis still to be done on the active crudes; bio-guided fractionation should also be conducted and may lead to the isolation of the major components in the active crude. Some species of *Fabaceae, Astaracea, Berberidaceae,Apiaceae* and *Lauraceaes* families extracts have anti-leishmanial activity and have been highly recommainded by many researches. Based on the findings of these studies, medicinal plants exhibited antileishmanial activity with inhibitory effect in both promastigotes and amastigotes forms of Leishmania. (Maryam Heidari-Kharaji *et al*. 2016).

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Table (1) Phytochemical analysis of *Ambrosia maritima* L. *(Asteraceae*) extracts.

plant	Phytochemical composition													
	alkaloids				**Flavonoids:**			**Saponins**	**Tri-terpenes**		**Tannins**	**carbohydrates**		**Amino acid**
	Maeyar's test	Wagner's test	Dragendorff's test.	KOH test	NH_3OH test.	$AlCl_3$ 10% test	Shinoda test	Foam test	Salkowski test	Liebermann - Burcha-rd test	Ferric chloride test	Molisch's test:	Fehling's / Benedict's test	Ninhydrin test
A. maritima L	+ + +	+ + +	+ + +	–	–	–	–	+	+ + +	+ + +	+ + +	+ +	+	+

+: present in low ++: present in moderate , +++: present in high , - : absent

Cytotoxicity screening:

Table (2): Cytotoxicity of plant extracts on normal cell lines (L20B) as measured by the MTT assay:

NO	Name of Extracts	Concentration (µg/ml)			IC_{50} (µg/ml)	IC_{50}
		Inhibition (%) ± SD				
		500	250	125		
1	*Ambrosia maritima* L(Demssisa)	45.71 ± 0.02	39.14 ± 0.06	23.43 ± 0.05	567.2454	> 100
3	*Control	96.28 ± 0.01				< 30

Key: IC_{50} < 30 µg/ml: high toxic, > 100 µg/ml: no toxic *Control = Triton-x100 was used as the control positive at 0.2 µg/mL.

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